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# Population Variability of Rotylenchulus reniformis in Cotton Agroecosystems

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# POPULATION VARIABILITY OF ROTYLENCHULUS RENIFORMIS IN COTTON AGROECOSYSTEMS

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Plant and Environmental Sciences

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by
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## ABSTRACT

*Rotylenchulus reniformis*, reniform nematode, is a highly variable species and an economically important pest in many cotton fields across the southeast. Rotation to resistant or poor host crops is a prescribed method for management of reniform nematode. An increase in the incidence and prevalence of the nematode in the United States has been reported over the last two decades. However it is not clear whether the observed increase is related to the emergence of novel populations that are more aggressive or have a higher fitness or shifts in host availability or susceptibility. The objectives of this research were to determine the variability of the species by studying the effect of crop rotations on the genotype of reniform nematode populations, the effect of temperature on the embryonic development of reniform nematode populations from the southeastern United States, and to determine the genetic diversity of *R. reniformis* populations representing cotton-growing areas in the United States. We used AFLPs (Amplified Fragment Length Polymorphisms) to determine changes in population structure due to rotations. Six rotation schemes were used that included susceptible cotton and soybean, resistant soybean, and non-host corn during four planting cycles. Distinct changes in genotype were observed following rotations with resistant soybean or corn. Rotations involving reniform nematode resistant and susceptible soybean cultivars had the most distinct effect on population structure. To determine variability of populations in the absence of a host, embryogenesis was compared among three geographic reniform nematode populations at 20, 25, 30, and 35ºC. The greatest differences among populations occurred at 20 and 35ºC. Results at the intermediate

temperatures (25 and 30$^{o}$C) were similar for the three populations. Reniform nematode may have the ability to increase its distribution range through variants able to reproduce in a wider temperature range, but genetic variability of isolates needs to be determined. To determine the genetic variability of individual reniform nematodes we developed and optimized 10 polymorphic microsatellite loci and tested loci on 160 individual reniform nematodes to determine informative genetic variation of isolates from the Southeastern United States, Colombia, and Japan and *R. parvus* isolated from a field in Arizona. Most isolates exhibited significant genotypic differentiation and moderate to very high genetic differentiation based on $F_{ST}$ analysis. The most consistently differentiated isolates were found reproducing parthenogenetically in Japan. Understanding the adaptive potential of this species is crucial for the development of durable management strategies, and our results indicate a need for an standardized method to genetically characterize *R. reniformis* variants and monitor variation.

# DEDICATION

This dissertation is dedicated to everyone who has loved and supported me while I worked on obtaining my PhD, especially my family. My family is the driving force in my life that has led to me wanting to learn more and achieve more. Without their love and support, none of this would have been possible. I would like to make a special dedication to my parents. If it was not for their love and financial support, I would not have had enough faith in myself to be so successful in life. I look forward to many more years of health, happiness, and love.

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I wish to also thank all of the members of the Clemson University Nematology Lab past, present, and future. I will always be proud to be associated with Clemson University and a lab headed by Dr. Paula Agudelo. No matter where life leads everyone, this common thread will always be there to connect us.

Finally, I would like to thank the faculty, staff, and students of the Department of Entomology, Soils, and Plant Sciences and the Plant and Environmental Sciences Program. It was an honor to represent both during my graduate career.

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# CHAPTER ONE

# EFFECT OF CROP ROTATIONS ON ROTYLENCHULUS RENIFORMIS POPULATION STRUCTURE

*Rotylenchulus reniformis* (reniform nematode) can parasitize over 300 plant species including soybean and cotton (Robinson et al., 1997). In the United States, reniform nematode is considered a major nematode pest of Upland cotton (*Gossypium hirsutum*), causing an estimated loss of 203,720 bales due to yield decrease (Blasingame et al., 2010) in 2009. In the same year, reniform nematode was the primary nematode pest of Upland Cotton in Alabama, Louisiana, Mississippi, and Tennessee (Blasingame et al., 2010) causing greater losses than root-knot nematodes for cotton producers in these states.

Use of nematicides for reniform nematode reduction can be financially and environmentally costly. Prohibition of chemical nematicide use is increasing in a number of countries due to adverse environmental effects (Abawi & Widmer, 2000). Alternatives to nematicides include using soil amendments, host plant resistance, cover cropping, and crop rotations (Gaur & Perry, 1991). Increasing levels of host plant resistance has become a preferred method of controlling yield losses due to plant-parasitic nematodes. Resistance to reniform nematode has not been found to naturally occur in Upland Cotton (Usery et al., 2005; Robinson et al., 1999). Resistance has been introgressed from *Gossypium longicalyx* (Robinson et al., 2007), but there is currently no commercially available cotton cultivar with resistance to reniform nematode.

Crop rotations to corn (*Zea mays*), a poor host for reniform nematode (Davis and Webster, 2005) or a resistant soybean (*Glycine max*) cultivar are a prescribed method of control for cotton producers in Georgia, North Carolina, and Tennessee (Brown et al., 2008; Koenning, 2010; Newman, 2010). Several studies have looked at the effect of crop rotations on reniform nematode reproduction. Results showed that rotations suppressed reniform nematode population development compared to continuous cotton (Davis et al., 2003; Plunkett et al., 2002, 2003; Gazaway, 1998, 2000; Stetina et al., 2007). Cotton yields have also been higher when plots are rotated to corn or resistant soybean versus plots planted with continuous cotton in North Carolina, Georgia, Arkansas, and Alabama (Davis et al., 2003; Plunkett et al., 2002, 2003; Gazaway, 1998, 2000; Stetina et al., 2007). Corn and resistant soybean can decrease reniform nematode population numbers in the year they are grown, but sharp increases in numbers have been reported when cotton is returned as a host (Plunkett, 2003; Gazaway et al., 1998, 2000). Researchers have found no significant differences among cotton cultivars or corn hybrids used in rotations suggesting it is a species effect and not dependent on cultivar (Stetina, 2007).

Despite the potential for adaptation, differentiation, and spread of a resistance allele among expanding populations of reniform nematodes, the effect of crop rotations on reniform nematode population structure has not been studied. Use of resistant cultivars or poor hosts may provide a method of nematode control that imposes a selective pressure on nematodes allowing a population to overcome host plant resistance (Muller, 1992; Turner & Fleming, 2002). Amplified Fragment Length Polymorphisms (AFLPs) are a method for genotyping that allows for analysis of an entire genome

without prior sequence information (Vos et al., 1995) and is a useful tool for revealing genetic diversity within a species. AFLPs can allow for the analysis of population structure changes induced by the plant host. Our objective was to determine the effect of crop rotations on population structure of reniform nematode using AFLP markers as a step towards understanding the interactions between genetic connectivity and the mediation of reniform nematode population expansion.

## Materials and Methods

*Crop Rotations:* Crop rotations were simulated in a greenhouse. Field soil infested with *R. reniformis* was obtained from a cotton field located near St. Matthews, SC. An aliquot of 1,000 individuals extracted from the infested field soil served as the baseline for all genotype comparisons. Other nematode species were present in the field soil, but reniform nematode was the predominant species present. The field soil was mixed with pasteurized loamy sand in a 38-liter plastic container. The initial inoculum level of reniform nematode was calculated to be ~3,000 vermiform *R. reniformis* per 200 cc. For each rotation, four plants were planted per container. A total of six rotation schemes were used to analyze the effect of host rotations on predominant reniform nematode genotype. Rotations included: 1) cotton, corn, cotton, corn; 2) susceptible soybean, corn, susceptible soybean, corn; 3) resistant soybean, cotton, resistant soybean, cotton; 4) corn, cotton, corn, cotton; 5) continuous susceptible soybean; 6) continuous cotton. Cotton cultivar 'Deltapine 50' (PI 529566), corn hybrid Funk's Waxy (PI 504055), and soybean cultivars 'Braxton' (susceptible) (PI 548659) and 'Forrest'

(resistant) (PI548655) were used. Each cycle allowed for reproduction over a 120 day period. After 120 days, the plants were destroyed and 4 soil samples of ~200 cc were collected from each rotation and vermiform reniform nematodes were counted. The soil that was removed for nematode counts was replaced with pasteurized loamy sand. The next cycle was planted directly into the designated rotation container for a total of 4 cycles.

*DNA extraction:* Following each rotation cycle all nematode counts were combined and 4 aliquots of 1,000 vermiform reniform nematodes were transferred into 1.5 ml tubes. Nematodes from all 4 counts were combined to remove any effect of specific plant genotype that is not indicative of the species or variety as a whole. DNA was extracted from nematode aliquots using E.Z. N.A. ™ Mag-Bind™ Blood DNA Kit (Omega Bio-Tek) with a slight modification to manufacturer's protocol. Prior to adding Elution Buffer in step 1, nematodes were frozen in liquid nitrogen then crushed with a pestle. The remaining steps followed the manufacturer's protocol except for step 20. Instead of adding 100-200 µl Elution Buffer, only 50 µl was added to increase DNA concentration. DNA was stored at -20°C.

*AFLP Amplification:* AFLP analysis was performed using AFLP® Analysis System I (Invitrogen). To begin 10 µl of DNA was used for restriction digestion with *Eco*R I and *Mse* I enzymes and followed manufacturer's protocol. A slight modification was implemented during pre-amplification reactions. Instead of 20 cycles for pre-amplification PCR, 25 cycles were performed. Pre-amplification PCR reactions were

performed in a PCR Sprint thermocycler. After pre-amplification PCR, instead of performing a 1:50 dilution of PCR product, a 1:20 dilution was used to increase product concentration for selective amplification. Selective amplification was performed using 4 primer pairs each with a fluorescently labeled forward primer. Selective nucleotides on primer pairs was as follows: 1) E-AAG, M-CTA; 2) E-ACA, M-CAA; 3)E-ACA, M-CAC; 4) E-ACG, MCTC. Fragments were sequenced using an ABI3730xl DNA Analyzer and visualized using GeneMapper V 4.0. GeneMapper was used to construct a genotype for samples consisting of a binary system of 0 for allele absence and 1 for allele presence.

*Data Analysis:* Genotypes produced from GeneMapper were analyzed for the best fit population grouping number using the genotype assignment program STRUCTURE V 2.3. Genotypes from four primer pairs were combined per sample to give a higher resolution image of predominant genotype following rotation cycles. An initial burn-in period of 200,000 iterations was used followed by 500,000 MCMC iterations for all runs of STRUCTURE. Models were altered to determine the best fit model to analyze each rotation scheme.

Results

The population densities observed after each rotation cycle behaved as expected (Figure 1.1). Population densities in soil were lowest after resistant soybean cv. Forrest (ranged from 495 to 1,470/200 cc) or after corn (ranged from 116 to 3,175/200 cc). Population densities were highest after cotton (ranged from 1,879 to 9,440/200 cc) or

susceptible soybean cv. Braxton (ranged from 2,153 to 10,378/200 cc) was returned as the host.

From AFLP analysis, 279 polymorphic bands were observed from 4 primer combinations with a total of 6 selective nucleotides in each primer pair. From analyses of population grouping assignments in STRUCTURE V 2.3, the best fit model for all rotations was an admixture model with allele frequencies correlated. The admixture model allows the program to look at each genotype as having ancestry from multiple clusters and each individual may not belong to a single cluster. Correlated allele frequency models allows for analysis when genotypes may be quite similar and can improve clustering for closely related populations. The value of population cluster, K, was varied from 1 to 6 for each rotation scheme and run several times to confirm that results across multiple runs were similar. The best fit number of clusters or population structures was determined by the value of K (Table 1.1) with the highest log probability (lnP(D)). In STRUCTURE, K represents the number of populations which are characterized by a set of allele frequencies at all loci. Individuals are assigned to clusters in which allele frequencies are similar.

The rotation scheme of continuous susceptible soybean cv. Braxton (BBBB) resulted in the best fit number of population clusters being 2 (K=2, lnP(D)=-1546.6). Genotypes were still similar to the original field genotype after four 120-day cycles (Figure 1.2A). The original field genotype was maintained through the four rotation cycles even though it was not always the dominant genotype (ex. Cycle 2). No distinct

effect on genotype was observed from host selection pressure when only Braxton was used as a host.

The rotation scheme consisting of continuous cotton (DDDD) maintained a high level of diversity throughout the experiment and resulted in the best fit number of population clusters being 3 (K=3, lnP(D)=-1623.8). Portions similar to the original field genotype were maintained throughout the 4 cycles which was also observed from the other continuous susceptible host rotation scheme BBBB (Figure 1.2E). However, there was more diversity present when cotton is used as a continuous host compared to susceptible soybean as a continuous host. Rotation DDDD did not have a distinct genotype between all 4 replicates following each cycle. Portions of each genotype following all cycles were similar to previous and post cycle genotypes as well as similar to the original field genotype. Continuous cotton does not appear to exert host selection pressure on reniform nematode after four 120 day cycles but allows for more diversity in genotype than continuous susceptible soybean.

The rotation scheme alternating susceptible soybean cv. Braxton with corn (BCBC) had a best fit number of population clusters of 5 (K=5, lnP(D)=-1520.1). After the first rotation, genotypes from 3 replicates were still similar to the original field genotype (Figure 1.2B). Only 3 replicates were possible due to DNA extraction being insufficient for AFLP analysis from the fourth aliquot of 1,000 reniform nematodes. When corn was the host during cycle 2, a portion of the genotype from the original field genotype was still detectable in all 4 replicates (~5.5%). A portion of the original field

genotype was not detectable in the following 2 cycles. When Braxton was again returned as the host, 100% of the genotypes from all 4 replicates clustered similarly and were not similar to any other genotypes found in the BCBC rotation. This result was again observed when corn was returned as the host in cycle 4. Cycles 2 through 4 each resulted in a distinctly unique clustering of genotypes that was not observed after cycle 1. The field genotype and cycle 1 genotype were not detectable in cycle 3 when Braxton was returned as the host. When corn was introduced as the host, distinct population structuring began to occur in the remaining cycles.

The rotation scheme alternating the resistant soybean cv. Forrest with cotton (FDFD) had a best fit number of population clusters of 4 (K=4, lnP(D)=-980.2). No genotypes were able to be recovered following cycle 1 due to low recovery of vermiform reniform nematodes. Only 2 replicates were recovered after cycle 3 but 4 replicates were recovered when cotton was the host (cycles 2 and 4). Following each rotation cycle, replicates able to be collected had nearly 100% homogeneity in genotypes following each rotation cycle (Figure 1.2E). No cycles clustered similarly. The percentage of similarity to the original field genotype after cycle 1 could not be determined, but genotypes from cycle 2 and 3 had less than 0.5% homogeneity with original field genotype. No proportion of the original field genotype was observed in cycle 4. In cycles 2 and 4, when cotton was the host, genotypes exhibited 0% homogeneity between genotypes from the two cycles. The predominant genotype following cotton as a host in cycle 2 was not maintained and was not detectable after cotton was returned as a host in cycle 4 following resistant soybean as the host.

The rotation scheme alternating cotton with corn (DCDC) had a best fit number of population clusters of 5 (K=5, lnP(D)=-1248.6). Presence of the original field genotype was not detectable when corn was the host in cycles 2 and 4 but was detectable when cotton was the host in cycle 1 and was then again detectable when reniform nematode numbers were increased in cycle 3 (Figure 1.2D). When corn was the host there was 100% homogeneity between replicate genotypes. Only 2 replicates were able to be recovered following cycle 4. The 2 replicates recovered following cycle 4 were similar to a portion of the genotype from cycle 3 (25.9%, 29.9%, 38.0%, and 7.9% homogeneity). The predominant genotype following cycle 1 was not present in the following 3 cycles after the population had corn as a host. The predominant genotype following cycle 3 was not homogeneous with any other cycle's predominant genotype but diversity was still maintained when cotton was the host.

A different result was observed when corn was the first host followed by cotton (CDCD). Here the best fit number of population clusters was 3 (K=3, lnP(D)=-1480.0). Only one replicate was able to be recovered following corn as a host in cycles 1 and 3. There was 100% homogeneity of genotypes following cycles 1 and 3 with the original field genotype (Figure 1.2C). A portion of the field genotype was still detectable after cycle 4 in two of the 4 replicates. This suggests that there was an effect of rotation sequence on predominant genotypes. The similarity between cycles 1 and 3 with the field genotype suggests that the majority of individuals recovered were not reproducing but remaining viable in the soil until a suitable host was returned.

Discussion

No previous study has looked at the effect of crop rotations on the predominant genotype of reniform nematode. Our results showed there is an effect of crop rotation on population structure of reniform nematode. The most distinct effects were observed when resistant or susceptible soybean was used in a rotation scheme. Continuous cotton cropping appears to maintain diversity of population specific genotypes but does allow for genetic drift from the original field genotype. Continuous soybean maintains the original field genotype for a longer period of time and may be more useful for maintaining field genotype when culturing reniform nematodes in a greenhouse. When cotton is used in rotation with resistant soybean cv. Forrest, replicates from individual cycles were nearly 100% homogenous (Figure 1.2F). The return of cotton as a host in cycle 4 did not result in a genotype that was similar to the genotype when cotton was the previous host in cycle 2. The field genotype was nearly undetectable after 2 cycles of Forrest followed by cotton. Differences were observed between CDCD (Figure 1.2C) and DCDC (Figure 1.2E) suggesting that the rotation scheme does have different effects on genotype. In DCDC, predominant genotypes following cotton as a host in cycle 1 were similar but only a small portion of the field genotype was detectable. Increases in nematode population density (Figure 1.1) show that nematodes were reproducing and the predominant genotype after 120 days was not similar to the original field genotype from genetic recombination. In contrast, CDCD genotypes from cycles 1 and 3 were similar to the field genotype suggesting that nematodes able to remain viable in the soil were the only ones recovered.

Similar results were found by Kaplan et al. (1999) when studying host effect on *Heterodera schachtii* isolates. In their study, molecular markers (AFLPs and RAPDs) revealed several distinct genetic polymorphisms between 3 *H. schachtii* isolates subjected to two 80-day host sequences involving poor, moderate, and good hosts. Genetic polymorphisms for a population resulted from different host plants but also depended on the genotype of the initial population and host on which nematodes had been reared. Results showed rapid host-induced selection, which we also observed in our study with *R. reniformis*. Genotypic changes in nematode populations can be induced by plants of differing host suitability which is similar to our results of genotype altering following crop rotations with resistant or poor hosts. Results for *H. schachtii* showed different geographic isolates responded differently to selection pressure exerted by particular hosts. Future research for *R. reniformis* would need to include various geographic isolates to determine whether response to host selection pressure is similar across the species or varies by genotype.

Genetic variability of isolates in response to crop rotations needs to be studied in order to understand an isolate's response to selection pressure. Research aimed at identifying molecular markers able to discriminate population specific responses to crop rotations or use of host resistance can help to monitor or detect reniform nematode isolate variation. Understanding population response can help to extend the durability of resistance genes in host plants and avoid emergence of isolates able to reproduce on what has traditionally been considered a non-host or poor host. Genetic differentiation in host associated forms of parasites has been reported for plant feeding insects. In the

snakeweed grasshopper (*Hesperotettix viridis*), a genetically distinct lineage associated with two host plants, *Solidago mollis* and *Gutierrezia sarothrae*, has been accompanied by substantial genetic differentiation between the host associated forms. Genetic differentiation between the host associated forms is also maintained even when the two co-occur at the same site. Data suggests that lineages arose from a single ancestral host shift as opposed to local adaptation and subsequent genetic differentiation (Sword et al., 2005).

AFLP markers have proven to be a useful molecular tool for characterizing variants in fungi and nematodes. They can be used for differentiating host specific races is various parasitic organisms. In *Monilinia laxa*, AFLP analysis showed isolates were correlated to host genus and not to geographic origin (Gril et al., 2008). These findings also genetically supported the specialization of isolates from apple trees forming the *forma specialis* taxonomic group *mali* and are adapted to the specific host apple trees. Two forms of the disease net blotch of barley, *Pyrenophora teres f. teres* and *P. teres f. maculata*, were molecularly distinguished using AFLP markers even though the two groups are morphologically similar but cause different symptoms (Lehmensiek et al., 2010). AFLP analysis was able to distinguish between different host races of the tobacco cyst nematode complex (Marché et al., 2001). Markers were identified that distinguished two of the tobacco cyst-nematode subgroups, *Globodera tabacum tabacum* and *Globodera tabacum solanacearum*, even when the two had the same geographic origin. In *Ditylenchus dipsaci*, the stem and bulb nematode, thirty biological races have been described according to their host preferences but are morphologically similar

(Sturhan & Brzeski, 1991; Janssen, 1994). The only race that could be distinguished morphologically from its greater body size was more harmful to *Vicia faba* (faba bean) and has been termed a "giant" type in contrast to "normal" types. AFLP analysis showed clear distinction between giant and normal populations of *D. dipsaci* suggesting that they should be considered as distinct species (Esquibet et al., 2003).

Selection for host specific pathotypes able to break resistance may result from the use of crop rotations and can lead to the need for a race schematic to characterize reniform nematode variants. AFLP markers may prove to be a useful molecular tool for characterizing variants and monitoring race formations with varying virulence on host species. Population responses to host plants will assist in crop-mediated management options for aid in identifying stable resistance genes. Assessment of long term host induced selection in the field can help determine whether markers are useful in resistance management. This is the first report of host effect on population structure of *R. reniformis* following prescribed crop rotations.

**Table 1.1.** Six rotation schemes used to determine host selection pressure on *Rotylenchulus reniformis*, rotation abbreviation, number of distinct population groupings (K) inferred using genotype assignment tests implemented in STRUCTURE V 2.3, and log probability(lnP(D)) of results from STRUCTURE V 2.3 analysis.

| Rotation Scheme | Abbreviation | K | LnP(D) |
|---|---|---|---|
| Braxton*-Braxton | BBBB | 2 | -1546.6 |
| Braxton-Corn | BCBC | 5 | -1520.1 |
| Corn-Cotton | CDCD | 3 | -1480.0 |
| Cotton-Corn | DCDC | 5 | -1248.6 |
| Cotton-Cotton | DDDD | 3 | -1623.8 |
| Forrest**-Cotton | FDFD | 4 | -980.2 |

*Braxton is susceptible soybean cultivar for *R. reniformis*.

**Forrest is a resistant soybean cultivar for *R. reniformis*.

**Figure 1.1.** Mean reniform nematode counts of 4 soil samples of ~200 cc each following 4 crop cycles for 6 rotation schemes.

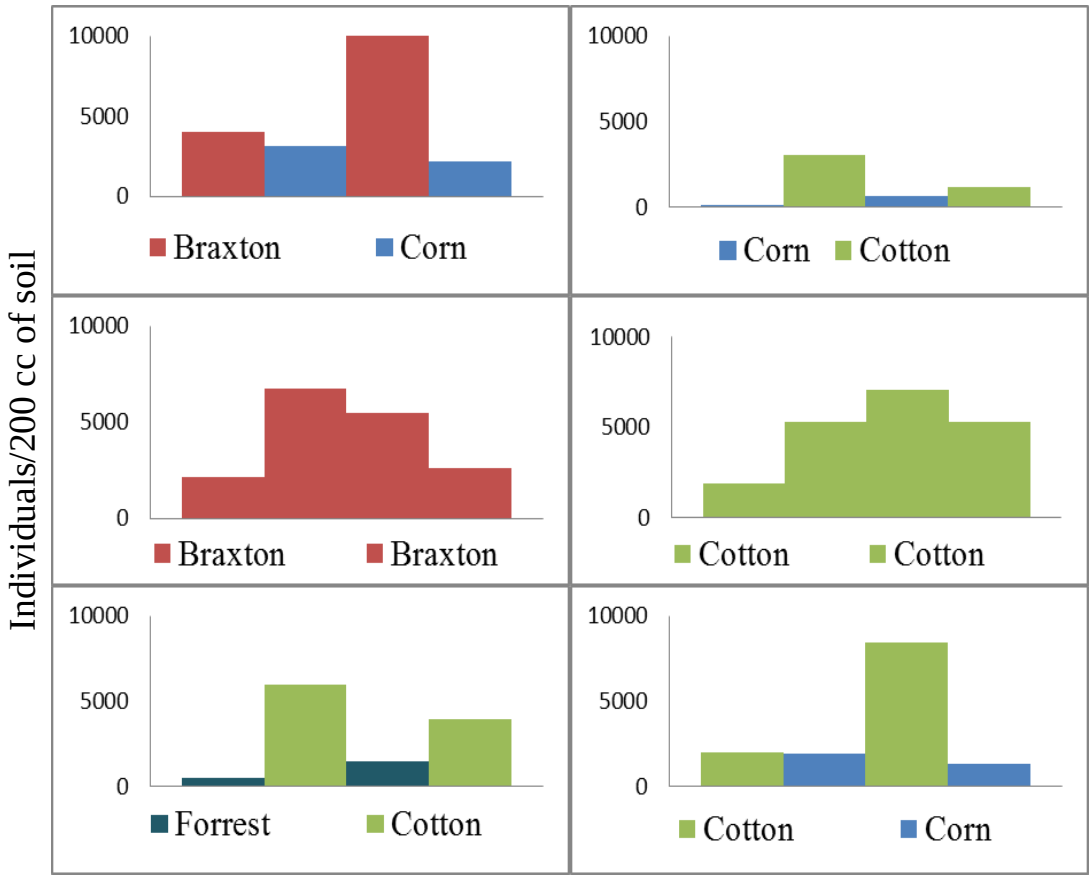
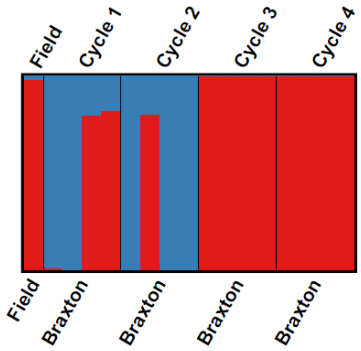
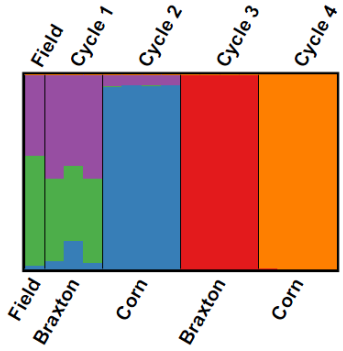


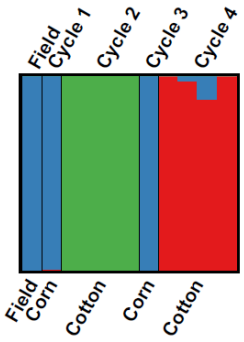
**Figure 1.2**. Graphical representation of *R. reniformis* genotypes following four crop rotation cycles using six different rotation schemes produced from STRUCTURE V 2.3 with each line representing the genotype of an aliquot of 1,000 reniform nematodes using 4 selective primer pairs with 6 selective nucleotides each.



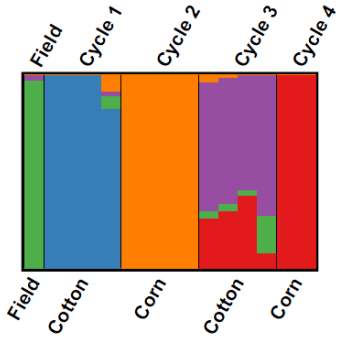

(A) BBBB



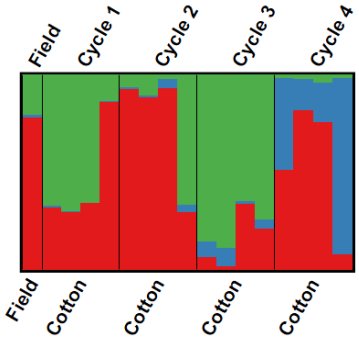

(B) BCBC



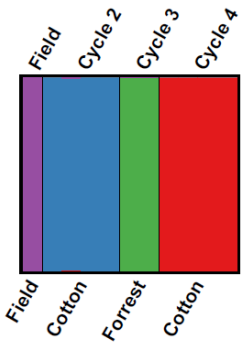

(C) CDCD




(D) DCDC




(E) DDDD




(F) FDFD

# CHAPTER TWO

# EFFECT OF TEMPERATURE ON THE EMBRYOGENESIS OF GEOGRAPHIC POPULATIONS OF ROTYLENCHULUS RENIFORMIS

*Rotylenchulus reniformis* is a broadly distributed species in tropical, subtropical, and some temperate regions of the world. It affects a large number of cultivated plants (Robinson et al., 1997; Gaur & Perry, 1991) as an obligate, sedentary, semi-endoparasite of roots. The life cycle of *R. reniformis* was initially described by Linford and Oliveira (1940). Mature females lay one-celled eggs into a gelatinous matrix, where the embryo develops into a first-stage juvenile (J1). The first cuticle molt occurs while the nematode is still in the egg. The second-stage juvenile (J2) emerges from the egg, and subsequent juvenile stages (J3 and J4) remain in the soil until adulthood is reached (Robinson et al., 1997). Eggs are able to hatch in water without the influence of a host plant and juveniles will develop into males and pre-adult females without feeding.

Cotton crop specialists and nematologists report an increase in the distribution and prevalence of reniform nematode in the United States over the last decade (Robinson, 2007). Its incidence has been documented to be on the rise in the southeastern cotton-growing areas and it is considered to have replaced root-knot nematode (*Meloidogyne incognita*) as the major nematode pest of cotton in Mississippi, Louisiana, and Alabama (Robinson, 2007). It is unknown whether this observed increase in importance is related to the emergence of novel, more aggressive populations, or of populations with increased fitness possibly due to changes in cropping histories or the host susceptibility of prevalent

cultivars. Although the life history and some management aspects of this nematode are known, most of its basic biology remains to be investigated.

The effects of temperature on embryonic development may comprise an important factor in the ecology and distribution of reniform nematode. The thermal time requirements for embryogenesis in reniform nematode are not known. Sivakumar and Seshadri (1971) studied the embryonic development of this nematode in detail, reporting that eggs develop into second-stage juveniles in six to seven days, but did not include any temperature information in their study. The time necessary for reniform nematode to complete its life cycle has been shown to vary with temperature and host species (Rebois, 1973; Heald & Inserra, 1988). Differences between populations in their sensitivity to temperature have also been observed (Heald & Inserra, 1988). However, there are no studies on the effect of temperature on the embryogenesis of this organism. The thermal requirement for embryonic development can be very high in plant-parasitic nematodes. For example, in *Meloidogyne javanica*, it constitutes 40% of the energy required for development to maturity (Trudgill, 1995a). This understudied aspect of reniform nematode biology could be an important factor in the overall fitness of the nematode and its ability to extend its distribution range. The objective of this study was to compare the effect of temperature on the embryogenesis of three populations of reniform nematode isolated from cotton fields in the southeastern United States.

## Materials and Methods

Reniform nematode populations were obtained from cotton fields in Huxford, Alabama (AL), Glendora, Mississippi (MS), and St. Matthews, South Carolina (SC). These populations were morphologically indistinguishable (Agudelo et al., 2005). Infested field soil was used to increase the nematodes in the greenhouse on soybean (*Glycine max*) cv. 'Braxton'. Seeds were germinated in a germination mix and transferred to one-gallon plastic containers after the first true leaves emerged. Three to four plants were maintained in each container holding infested field soil mixed with a pasteurized sandy loam. The populations were maintained separately for two months prior to the beginning of this study.

After 60 days, infected roots were carefully washed to remove soil particles. Egg masses were removed from roots and gently crushed to release eggs. Individual freshly-laid eggs, in the one-celled stage, were arbitrarily selected and transferred to wells of a 24-well plate (VWR, Polystyrene) containing 1 ml of 0.5% water agar. The plates were sealed with Parafilm and incubated in the dark at 20, 25, 30, and 35ºC ± 0.5ºC. The eggs in each well were examined twice daily to monitor development of the embryo until eclosion of the second stage juveniles. A 24-hour period was added to all data in order to account for the time elapsed between eggs being laid and the transfer to the plates. A minimum of 20 replicates were used for all data averages presented in this study.

The rate of development for each population was expressed as the reciprocal of the number of days required for completion of embryonic development. The cumulative hatch was calculated by successive addition of second-stage juveniles hatched each day

until the last eclosion and expressed as a percentage of the total observed. Data was analyzed with Statistical Analysis Software® (SAS, 2003, v9.1, Cary, NC) using the NLmixed procedure and a random effect for plate.

Results

*Average days to eclosion:* The time necessary for eclosion varied greatest among populations at the lowest (20°C) and the highest (35ºC) incubation temperatures (Table 2.1). Similar average days necessary for eclosion were observed for all populations at the two intermediate temperatures (25 and 30ºC). The three populations had an average of seven days to hatch at 25ºC. Both MS and SC had an average of 6 days to hatch at 30ºC, while AL had an average of 5 days at this incubation temperature. Both MS and SC had an average of 8 days to hatch at 20ºC, while AL averaged 10.7 days to hatch at this temperature. At 35ºC, AL and SC were similar with an average of 6.0 and 5.7 days to hatch, respectively. The population from Mississippi averaged 8 days to hatch at 35ºC.

*Average rate of development:* The average rate for egg development was the same for MS and SC at 30°C. At 30°C, AL had the same average rate of development as SC at 35°C. The rate of development was linear between 20 and 30ºC for all populations. SC was the only population to have a linear rate of development between 20 and 35°C. The rate of development for AL was slowest at 20°C and fastest at 30ºC. SC had the same rate of development at 35ºC as AL did at 30ºC.

*Cumulative hatch:* When the cumulative hatch was compared among populations and across temperatures, the largest variation occurred at 20 and 35ºC (Figure 2.1). For AL at 20ºC, the majority of eclosions occurred 12 days after egg laying. For MS, the majority of eclosions occurred between nine and ten days. SC had the fastest cumulative hatch with most eclosions occurring between eight and nine days. At 25ºC, the majority of eclosions for AL occurred at seven days, which was similar to MS and SC. Both MS and SC populations had the majority of eclosions occur between seven and eight days. The results for 30ºC were similar across all populations, with most eclosions occurring between five and six days. At 35ºC, AL had a similar percentage hatch to SC, with a peak of eclosion between five and six days. The MS population varied greatly from the AL and SC populations, having an eclosion peak between eight and nine days. The AL population had the longest development time to eclosion at 20°C, and 30°C was the best observed hatching temperature for this population. Hatching at 35ºC was faster than at 25ºC for this population. For MS, 30ºC was also the best observed temperature for embryogenesis, but 25ºC was more favorable for development than 35ºC. For SC, 35ºC was more favorable than 30ºC. For all populations, hatching at 25ºC and 30ºC was similar.

*SAS analysis:* A quadratic regression was used to model the time to eclosion data as a function of incubation temperature. Hypothesis tests for equality of regression functions across locations were conducted. The regression fits allowed for estimation of the optimal temperature associated with embryonic development. A significance level of 0.05 was used for all hypothesis tests. The predicted optimal temperatures for each

population were calculated: AL (31.4ºC), MS (28.4ºC), and SC (37.5ºC) ($P < 0.0001$) (Figure 2.2). There were significant differences ($P<0.0005$) among populations in their predicted optimal temperatures (inflection point). There was no significant difference between locations when incubation at 25 and 30ºC was compared. The effect of temperature was significant across locations ($P<0.0001$).

Discussion

We observed distinct effects of temperature on embryonic development of the three reniform nematode populations included in this study. The population isolated from a cotton field near Huxford, Alabama appears to be the most sensitive to lower temperatures, exhibiting the slowest development at 20°C. This behavior may be explained by the fact that winter temperatures in Huxford are normally 2-4$^{o}$C warmer than in the other two locations (Table 2.2). Heald and Inserra (1988) reported a differential sensitivity to low temperatures in a study comparing reproduction of reniform nematode populations from Louisiana, Texas, and Puerto Rico on lettuce. They found that the population from Puerto Rico was unable to reproduce on lettuce at 15$^{o}$C, and suggested that the populations from Texas and Louisiana were better adapted to cooler temperatures. The fastest rate of development for AL was observed at 30$^{o}$C and an optimal temperature of 31.4°C was calculated for this population. These temperatures are comparable to those reported by Rebois (1973) in his study of the effect of soil temperature on infection and reproduction of reniform nematode on soybean, where he found that fecundity was highest at 29.5$^{o}$C. However, in our study we have eliminated

the effects of temperature on the plant and all the indirect effects on nematode development through the interactions between the nematode and the plant. This allowed for a more accurate calculation of the optimal temperature for each reniform nematode population.

Of the three locations included in this study, Glendora, Mississippi has the lowest normal high temperatures (Table 2.2) consistently throughout the year, which may explain why the population from MS had the lowest calculated optimal temperature (28.4ºC) of the three. The behavior of the population from South Carolina is more difficult to explain because it exhibited the highest calculated optimal temperature (37.5ºC), but this location consistently has the lowest normal low temperatures of the three locations. We can only speculate that this population may have higher plasticity than the other two. Reniform nematode is known to be a highly polymorphic species, for which several phenotypic differences among geographic populations have been reported (Dasgupta et al., 1968; Nakasono, 2004; Agudelo et al., 2005).

Rebois (1973) reported that no nematode reproduction occurred when roots were grown at soil temperatures of 15 and 36ºC, and indicated that further research was needed to determine if the higher temperature restricted the host's ability to provide nutrients for the nematode or if there was a direct effect of temperature on the nematode. Our results suggest that embryonic development of reniform nematode is not stopped, or even significantly delayed, at 35°C. Furthermore, the population from South Carolina had the fastest rate of development at this temperature, and a calculated optimal temperature of

37.5°C. Our results indicate that eggs are capable of normal development at 36°C, and that the absence of nematode reproduction observed in other studies at this temperature may be through an effect on the plant and not the nematode.

The optimal temperature for embryogenesis of *Heterodera glycines* is 24$^{o}$C, with second- stage juveniles failing to emerge from cysts when incubated below 16$^{o}$C or above 36$^{o}$C (Alston & Schmitt, 1988). When compared with our results for reniform nematode, these temperatures correlate well with the more temperate distribution of soybean cyst nematode than reniform nematode in the United States. Bird (1972) reported that the optimal temperature for embryogenesis of *Meloidogyne javanica* lies between 25 and 30$^{o}$C, but he also noted that although embryogenesis is slightly faster at 30$^{o}$C, more eggs complete development at 25°C. Additionally, he observed that development was twice as fast at 25 and 30$^{o}$C than it was at 20$^{o}$C. Our reniform nematode population from Alabama behaved very similarly, developing twice as fast at 30°C (5.4 days on average) than at 20°C (10.7 days on average). We also observed faster development at 25 and 30°C than at 20°C for the other two populations, but the development time was not doubled.

Diez et al. (2003) showed that life-stage development of *M. incognita* was delayed in the presence of equal or higher number of *R. renifomis*, showing that *M. incognita* was more susceptible to antagonism by *R. reniformis* than the reverse. Temperature sensitivity may play a role in the explanation to the perceived displacement of *Meloidogyne incognita* by reniform nematode as the most important nematode pest of

cotton in Mississippi, Louisiana, and Alabama (Robinson, 2007). Other authors have discussed ecological advantages of reniform nematode over other nematode species, such as its ability to remain viable in dry soils over long periods of time (Gaur & Perry, 1991; Koenning, 2004; Robinson, 2007). The anhydrobiotic stage may extend the life cycle by up to two years, thus facilitating survival even when a suitable host is absent or allow the nematode to be dispersed long distances in dust storms or when farming equipment is moved from area to area.

In his review of host and plant temperature effects on nematode development rates and nematode ecology, Trudgill (1995b) discusses the ecological significance of linear relations between temperature and development rates. However, he also briefly mentions that several clearly non-linear relations have been demonstrated and cites examples in *Aphelenchus avenae* and *Xiphinema diversicaudatum*, among others. In contrast to the better known cases of these types of studies, i.e. for *Meloidogyne*, *Heterodera*, and *Globodera* species, where linear relations are described, we found a quadratic relationship between temperature and development rate for reniform nematode. This type of relation is less straight-forward to use for predictive purposes than a linear relationship.

Reniform nematode populations may have the ability to increase distribution range through variants able to reproduce in a wider temperature range, allowing for increased numbers in new areas. According to a report presented in 2000 by the US Global Change Research Program (USGCRP), average temperatures in the United States

have increased between 0.6°C and 2°C for the coastal Northeast, upper Midwest, and the Southwest over the past 100 years. The largest warming across the nation has occurred in winter. We do not have evidence to support that this increase in temperature has allowed the range of distribution for reniform nematode to expand and/or if it has provided longer seasons for increased reproduction. We do know, however, that reniform nematode populations present in the cotton-growing region of the United States have different abilities to respond to variations in temperature.

**Table 2.1.** Average days to eclosion for three geographic populations (Huxford, Alabama (AL), Glendora, Mississippi (MS), and Saint Matthews, South Carolina (SC)) at four incubation temperatures (20, 25, 30, 35ºC), with average rates, median, mode, range, standard deviation, and number of replicates at each temperature.

| Pop. | Temp. (°C) | Average[a] | Rate[b] ($days^{-1}$) | Median | Mode | Range | St. Dev. | Replicates |
|---|---|---|---|---|---|---|---|---|
| AL | 20 | 10.7 | 0.09 | 11 | 12 | 7-14 | 2.14 | 23 |
| | 25 | 7.1 | 0.14 | 7 | 7 | 6-9 | 0.09 | 24 |
| | 30 | 5.4 | 0.18 | 5 | 5 | 5-6 | 0.50 | 26 |
| | 35 | 6 | 0.13 | 6 | 6 | 5-8 | 1.02 | 25 |
| MS | 20 | 8.8 | 0.11 | 9 | 9 | 7-10 | 1.00 | 21 |
| | 25 | 7.3 | 0.14 | 8 | 8 | 6-8 | 0.76 | 24 |
| | 30 | 6.1 | 0.16 | 6 | 7 | 5-7 | 0.86 | 29 |
| | 35 | 8.1 | 0.12 | 9 | 8 | 6-11 | 1.35 | 20 |
| SC | 20 | 8.5 | 0.12 | 9 | 8 | 6-10 | 1.18 | 24 |
| | 25 | 7.2 | 0.14 | 7 | 8 | 6-8 | 0.82 | 39 |
| | 30 | 6.1 | 0.16 | 6 | 5 | 5-8 | 0.98 | 26 |
| | 35 | 5.7 | 0.18 | 6.5 | 6 | 5-7 | 0.80 | 20 |

[a] Average of days from single-cell egg to eclosion of second-stage juveniles.

[b] Average rate of development per day.

**Table 2.2.** Normal low and high temperatures for the locations from where the three populations included in this study were isolated.

(NOAA National Weather Service http://www.ncdc.noaa.gov/oa/ncdc.html)

| | Huxford, Alabama | | Glendora, Mississippi | | St. Matthews, South Carolina | |
|---|---|---|---|---|---|---|
| | Normal Low | Normal High | Normal Low | Normal High | Normal Low | Normal High |
| January | 3.3 | 15.6 | 1.1 | 11.7 | 0.5 | 13.3 |
| February | 5 | 18.3 | 3.3 | 15 | 1.7 | 16.1 |
| March | 8.9 | 21.7 | 7.2 | 19.4 | 6.1 | 20 |
| April | 11.7 | 25.6 | 11.1 | 23.9 | 9.4 | 24.4 |
| May | 16.7 | 29.4 | 16.7 | 28.3 | 14.4 | 28.3 |
| June | 20.6 | 32.2 | 20.6 | 32.2 | 18.9 | 31.7 |
| July | 21.7 | 33.3 | 22.2 | 33.9 | 21.1 | 33.3 |
| August | 21.7 | 33.3 | 21.7 | 33.3 | 20.6 | 32.8 |
| September | 19.4 | 31.7 | 18.3 | 30.6 | 17.8 | 30 |
| October | 12.2 | 27.2 | 11.7 | 25.6 | 10.6 | 25 |
| November | 8.3 | 21.7 | 6.7 | 18.9 | 6.1 | 20 |
| December | 5 | 17.2 | 2.8 | 13.9 | 1.7 | 15 |

**Figure 2.1.** Cumulative hatch of reniform nematode populations from three geographic locations (Alabama, Mississippi, and South Carolina) at four temperatures (20, 25, 30, and 35°C).

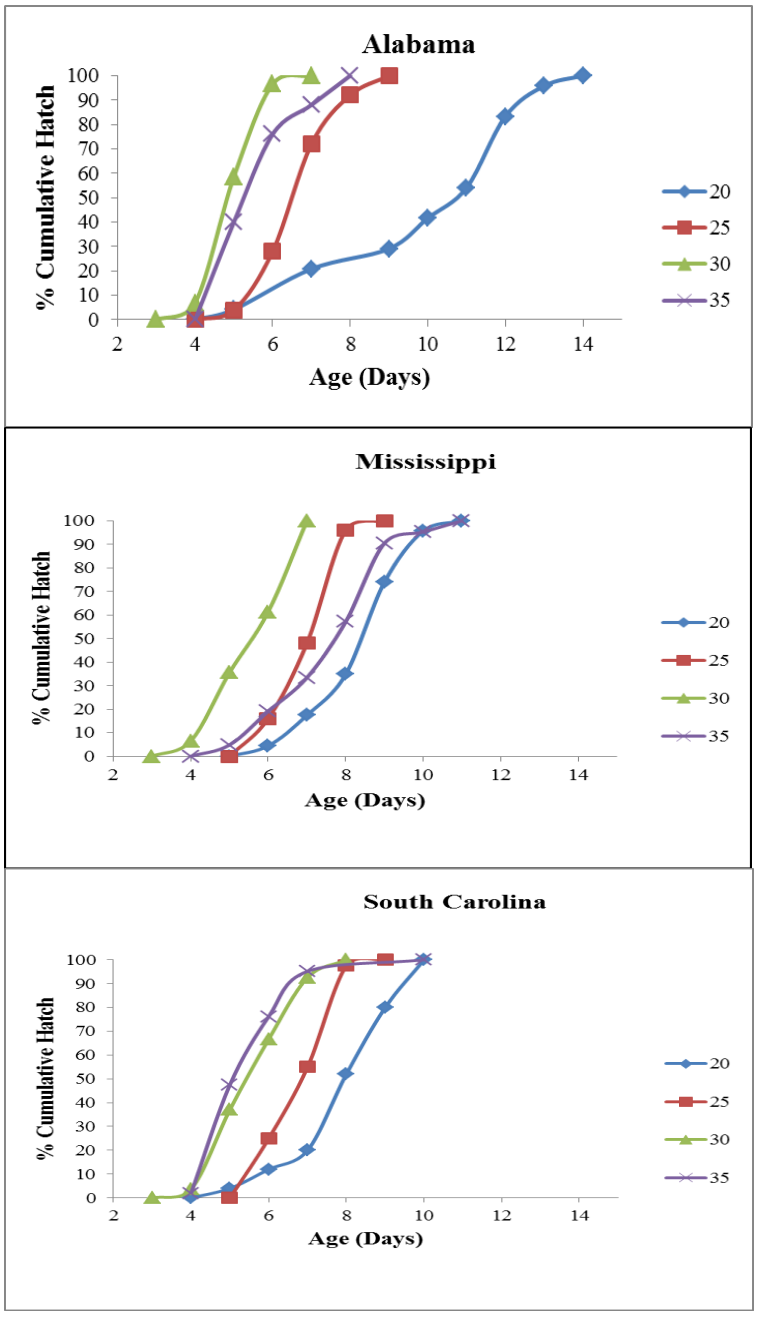
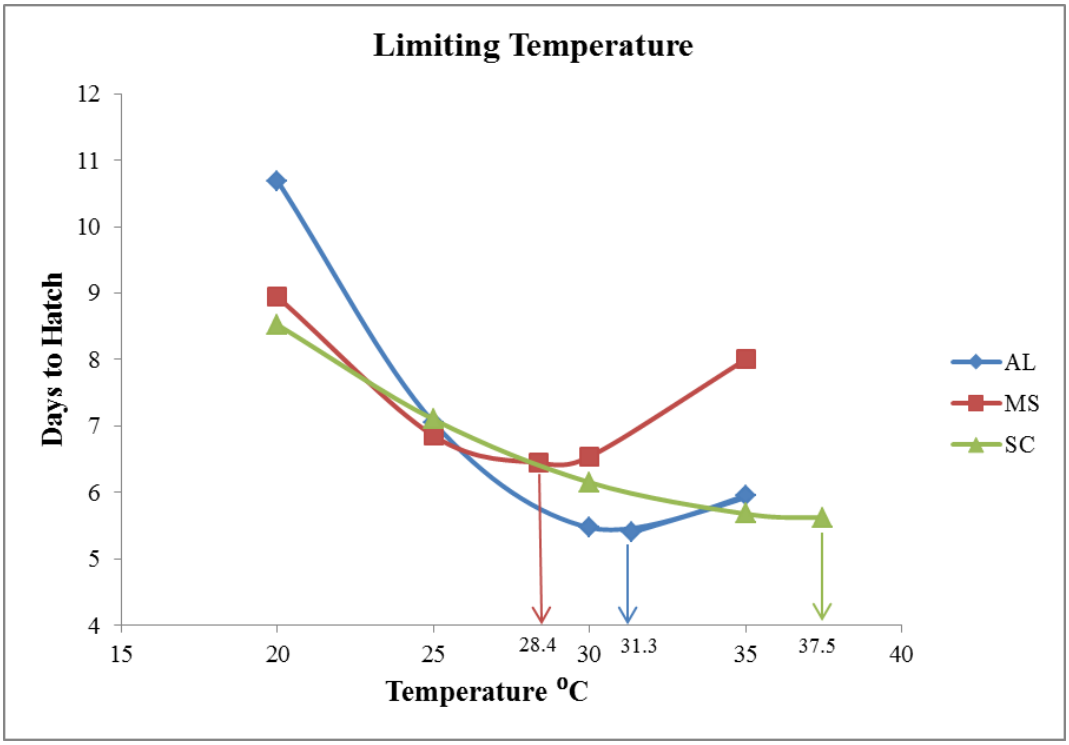


**Figure 2.2.** Quadratic regression fit to number of days to hatch against incubation temperatures for the three populations, Alabama (AL), Mississippi (MS), and South Carolina (SC).

AL: Days= 45.7833-2.5768*temp+0.0411*temp²

MS: Days= 35.0889-2.0185*temp+0.03556*temp²

SC: Days= 18.9654-0.7122*temp+0.009501*temp²



# CHAPTER THREE

# TEN POLYMORPHIC MICROSATELLITE LOCI FOR RENIFORM NEMATODE, ROTYLENCHULUS RENIFORMIS

*Rotylenchulus reniformis* (reniform nematode) is a sexually reproducing obligate, sedentary parasite of plants. It is distributed worldwide and can parasitize over 300 plant species (Robinson et al., 1997). Reniform nematode has been described as a highly phenotypically variable species (Leach et al., 2009, Agudelo et al., 2005, Nakasono, 2004) but little is known about genetic variation of the species, especially across loci within individuals. Arias et al. (2009) found genetic variability at 88 microsatellite loci in reniform nematode populations from four southern states (TX, LA, MS, GA), but the study used DNA pooled from 1,000-2,000 eggs rather than DNA from single individuals. Discussed is the development of microsatellite markers that can be used to test specific hypothesis regarding population dynamics among and within individual reniform nematode populations. The ability to examine alleles isolated from single individuals expands the use of these polymorphic, informative loci for studying population interactions due to refinement of heterozygosity and association among polymorphic sites within and between alleles across loci.

To develop microsatellite markers, reniform nematodes were obtained from infested field soil from Calhoun County, South Carolina and maintained in a greenhouse on cotton (*Gossypium hirsutum*) plants. DNA was extracted from ten vermiform *R. reniformis* using 25µl Worm Lysis Buffer (50mM KCl, 10mM Tris pH 8.3, 0.45% Tween 20, 0.45% Triton X, 60 µg/ml Proteinase K, 2.5mM $MgCl_2$, $dH_20$). One µl of extracted

DNA was used for Whole Genome Amplification (WGA) using GE Healthcare's GenomiPhi V2 kit. Microsatellite DNA loci were then isolated following the protocol established by Glenn and Schable (2005). After WGA, DNA was digested using enzymes BstU I or RSA I (New England Biolabs). Restricted DNA was ligated to double-stranded linkers to provide the primer binding site for subsequent PCR. Forward SuperSNX24 (5' –GTTTAAGGCCTAGCTAGCAGAATC-3') and reverse SuperSNX24+4P (5'-pGATTCTGCTAGCTAGGCCTTAAACAAAA-3') (IDT) were used. Ten µl of double stranded SuperSNX linkers was added to 15 µl of restricted DNA. A mixture of 3' biotinylated oligos [$(AAAC)_6$, $(AAAG)_6$, $(AATC)_6$, $(AATG)_6$, $(ACAG)_6$, $(ACCT)_6$, $(ACTC)_6$, $(ACTG)_6$] was used for enrichment of microsatellite containing DNA fragments. Microsatellite-enriched DNA was captured and removed using Dynabeads (Dynal) and amplified with SuperSNX-24 as a forward primer. DNA was cloned using Invitrogen's TOPO TA Cloning Kit with PCR 4-TOPO vector. Bacteria were plated on LB plates containing 50 µg/ml ampicillin. Transformed colonies were picked and amplified using an MJ Research PTC-100 thermocycler with M13 forward (-20) (5'-GTAAAACGACGGCCAG-3') and reverse (5'-CAGGAAACAGCTATGAC-3') primers. A 1% agarose gel was used to verify the presence of inserts. PCR products containing inserts within the 300-1000 bp range were purified and sequenced using an ABI 3130xl DNA analyser, located at Clemson University's Genomics Institute, using M13 forward and reverse primers (forward 5'-GTAAAACGACGGCCAG-3', reverse 3'-GGCTATGACCATG-5'). Sequences from forward and reverse reads were assembled and edited using BIOEDIT incorporating the

Clustal W alignment method. Mine SSR, a program available through the Clemson University Genomics Institute (http://www.genome.clemson.edu/resources/online_tools/ssr), was used to identify microsatellite repeats in contiguous sequences and to design primers.

Ten microsatellite loci developed proved to be polymorphic and were used to determine the genetic variability of individual reniform nematodes from the southeastern cotton growing region. DNA was extracted from a total of 100 individual reniform nematodes using Sigma Extract-N-Amp kit (XNAT2). In order to have a higher concentration of extracted DNA from individuals, a slight modification to the manufacturer's protocol was used. One nematode was placed into a 0.2 ml centrifuge tube containing 12.5 µl Extraction Solution. The nematode was then crushed using the tip of a <10 µl pipette tip. Subsequently 3.5 µl of Tissue Prep was added to each tube. Tubes were then mixed and centrifuged briefly to collect contents and incubated at 65 °C for 10 minutes followed by 95 °C for 3 minutes. Next, 12.5 µl of Neutralization Solution B was added to each tube, mixed, and centrifuged briefly. DNA from each individual was amplified at 10 microsatellite loci to determine genetic variability. Optimized annealing temperature was determined for each microsatellite primer pair using an MJ Research PTC-100 thermocycler. Cycling conditions were as follows: 94°C for 3 minutes followed by 35 cycles at 94°C for 30 seconds, optimized primer annealing temperature for 30 seconds, 72°C for one minute and a final extension at 72°C for 10 minutes. Each 20 µl PCR reaction contained 2 µl DNA and 18 µl PCR mix (1.25X PCR Buffer, 0.25 mM dNTPs, 2.5 mM $MgCl_2$, 0.5 µM Forward Primer, 0.5 µM Reverse

Primer, 0.125 U Taq). All forward primers were labeled with fluorescent dye FAM (Invitrogen). Amplicons were detected using an ABI 3730xl DNA Analyser with GeneScan 500 ROX used as the size standard (Applied Biosystems). Peak data was analyzed using Peak Scanner software version 1.0 (Applied Biosystems) and allele calls were visually inspected. The Excel Microsatellite Toolkit (Park, 2001) was used to calculate number of alleles per locus (k), observed and expected heterozygosities ($H_o$ and $H_e$), and polymorphic information content (PIC) (Table 3.1). MICROCHECKER version 2.2.3 (Van Oosterhout et al., 2004) was used to test for the presence of null alleles. Gametic disequilibrium and departures from Hardy-Weinberg proportions were determined using GenePop version 4.0 (Rousset, 2008). The dememorization number was 10000, the number of batches was 500 and the number of iterations per batch was 5000.

Expected and observed heterozygosities ranged from 0.244 to 0.889 and 0.071 to 0.600, respectively. PIC ranged from 0.238 to 0.874. Number of alleles ranged from 8 to 19 with an average of 12.8 alleles per locus. Null alleles were not detected. No gametic disequilibrium was detected between the 10 pair of loci for these 100 individuals. The *P*-value for gametic disequilibrium model tests ranged from 0.999968 to 0.147086. For GenePop the null hypothesis assumes that alleles at one locus are independent from alleles at another locus. Anything below a *P*-value of 0.05 would be considered in gametic disequilibrium. Out of the 10 loci, nine were determined to depart from Hardy-Weinberg proportions (*P*-value <0.00001). Here the null hypothesis assumes the random union of gametes and the alternative hypothesis was a deficiency of heterozygotes. Only

locus RR2-6 (*P*-value 0.2355) did not exhibit a departure from Hardy-Weinberg proportions. The nine loci that departed from Hardy-Weinberg proportions all had a high inbreeding coefficient ($F_{IS}$). Average $F_{IS}$ across all loci was 0.531. High levels of inbreeding are not surprising due to the low mobility of the species. This is the first report of genetic data supporting a high inbreeding coefficient for this species. Interestingly, this high frequency of inbreeding resulting in a high frequency of homozygosity occurs even with high level of genetic variation resulting in relatively large number of alleles per locus (Table 3.1). The combination of high homozygosity and relatively high genetic variation suggest that studies examining the rates and patterns of shared versus fixed alleles between populations will be possible and therefore these loci will be useful for studying reniform nematode population genetics.

**Table 3.1.** Primer sequences and characteristics of ten *Rotylenchulus reniformis* polymorphic microsatellite loci.

| Locus | Repeat motif | Primer sequence (5' → 3') | $T_a$ (°C) | $k$ | $H_e$ | $H_o$ | PIC | $F_{IS}$ | GenBank Accession no. |
|---|---|---|---|---|---|---|---|---|---|
| RR1-5 | $(ga)_7$, $(ga)_7$, $(ga)_7$, $(ga)_7$ | F:TGCGAGAGAGAGAG AGAGTGA R:TGGTGTTAATGGTGA AAGCG | 62 | 16 | 0.79 | 0.38 | 0.77 | 0.53 | HQ158019 |
| RR2-5 | $(cact)_3$ | F:GGGCACTGAAAACA CCAAGA R:TGAGTGGACACAAT CCGAAA | 60 | 10 | 0.38 | 0.07 | 0.36 | 0.82 | HQ158020 |
| RR2-6 | $(catt)_4$ | F:GAATGAATTGGCAA AATGGC R:CTCCATGGCTTTCTC CTCAG | 62 | 9 | 0.65 | 0.60 | 0.59 | 0.08 | HQ158021 |
| RR3-3 | $(catt)_3$, $(attc)_3$ | F:AATGGGCGTCTAGTT CCCTT R:ATCGAAAATGACAT GCACCA | 60 | 17 | 0.89 | 0.31 | 0.87 | 0.65 | HQ158013 |
| RR3-8 | $(ga)_{10}$, $(ag)_5$, $(ag)_5$ | F:TTGCTTTACTTCCGC TTCGT R:GCTCCATTTTTCCCA CTCAA | 61 | 14 | 0.76 | 0.53 | 0.72 | 0.30 | HQ158015 |
| RR4-1 | $(ttga)_4$ | F:AGAAAATGCTTTTCC CGAGG R:AGAATTAGCGTTTG GGGATG | 63 | 12 | 0.57 | 0.28 | 0.53 | 0.50 | HQ158016 |
| RR4-4 | $(ttga)_4$ | F:GAAAATGCTTTTCCC GAGGT R:CGGTAGAATTAGCG TTTGGG | 63 | 8 | 0.49 | 0.27 | 0.45 | 0.44 | HQ158017 |
| RR4-5 | $(tggg)_3$ | F:TGTAATGACCCGAGT GTGGA R:GGGTCTCCCGGTAGT ACGAT | 57 | 11 | 0.74 | 0.25 | 0.71 | 0.66 | HQ158018 |
| RR5 | $(ttg)_4$ | F:CTCCGCCATTACCAT TCTGT R:CCCCAATTCCAACA ACAATC | 54 | 19 | 0.87 | 0.15 | 0.86 | 0.83 | GU471239 |
| RR2 | $(tggg)_3$ | F:AAAGATGCCAAAAG AATGCG R:GATATGCGTTGGCTG TGTTG | 58 | 12 | 0.24 | 0.11 | 0.28 | 0.56 | GU471236 |

$T_a$ is the PCR annealing temperature; $k$ is the number of alleles observed at each locus; $H_e$ and $H_o$ are expected and observed heterozygosities, respectively, PIC is polymorphic information content at each locus, inbreeding coefficient ($F_{IS}$) and the GenBank Accession number.

# CHAPTER FOUR

# GENETIC VARIABILITY OF ROTYLENCHULUS RENIFORMIS

*Rotylenchulus reniformis* (reniform nematode) is a plant-parasitic nematode commonly associated with a large number of cultivated plants in subtropical, tropical, and temperate soils worldwide (Gaur & Perry, 1991; Nakasono, 2004; Robinson et al., 1997). In the United States, reniform nematode has become one of the major pests in Upland cotton (*Gossypium hirsutum*) production. Lint yield and quality can be significantly reduced due to reniform nematode infection (Cook et al., 1997). In 2009, an estimated 203,720 bales of cotton were lost due to reniform nematode in the United States (Blasingame et al., 2009). The highest losses occurred in Alabama, Louisiana, and Mississippi. Reniform nematode also caused greater loss than root-knot nematode in Alabama, Louisiana, Mississippi, and Tennessee. Cotton crop specialists have reported an increase in the distribution and prevalence of reniform nematode over the last decade, especially in the southeastern cotton-growing region (Robinson, 2007). In 2010, reniform nematode was found parasitizing cotton across the southeast, from Texas to Virginia. Resistance to reniform nematode has not been found naturally occurring in Upland Cotton (Robinson et al., 1999; Usery et al., 2005). Efforts to introgress resistance from cotton relatives, like *Gossypium longicalyx* (Robinson et al., 2007), are in progress, but there are currently no commercially available cotton cultivars with resistance to reniform nematode.

Reniform nematode has been described as a highly variable species. Morphological differences have been reported for isolates around the world, including populations from Japan (Nakasono, 2004), Brazil (Rosa et al., 2003), and Africa (Germani, 1978). Morphological differences have also been described among isolates from the southern cotton growing region (Agudelo et al., 2005). Differences in embryonic development were reported among isolates from cotton fields in Alabama, Mississippi, and South Carolina (Leach et al., 2009). One study in Japan found differences in the reproductive compatibility of isolates, reporting one isolate that reproduced strictly parthenogenetically (Nakasono, 2004). The genetic variability of this species has been understudied. Agudelo et al. (2005) found no polymorphisms in the ITS1 region among amphimictically reproducing individuals from the United States, Brazil, Colombia, Honduras, and Japan. Only the parthenogenetic isolate from Japan was distinct. In 2009, Arias et al. found genetic variability at 88 microsatellite loci from reniform populations from four southern states (Texas, Louisiana, Mississippi, and Georgia) using DNA pooled from 1,000 to 2,000 eggs, but the study did not look at genetic variation of individuals.

Currently there is no standardized method for characterizing phenotypic or genetic variants of *Rotylenchulus reniformis*. The development of effective management strategies is directly related to the ecological significance of the phenotypic variation in *R. reniformis* and the correlation of such variation with genetic diversity in the nematode. The identification of intraspecific variation will have practical significance to control

nematodes or reduce damage to crops through the use of resistant cultivars and non-host plants.

Microsatellites are repeating sequences of 1 to 6 base pairs that are highly polymorphic from variations present in number of repeats. They are also codominant and are useful in characterizing genetic variability of plant pathogens. Microsatellites have a fast rate of mutation accumulation and often follow an informative step-wise mutation model. Development of microsatellite markers to study individual nematode genomic DNA can allow for genetic comparison of individuals from populations throughout the cotton growing regions of the United States. Polymorphic microsatellite loci markers enable investigation of population subdivision, gene flow, parentage, and relatedness. The development of microsatellite markers for plant parasitic nematodes is often limited by low DNA recovery from individual nematodes. Glenn and Schable (2005) recommend using 2 to 3 µg of high quality DNA to begin microsatellite marker development. Thousands of nematodes are required to achieve this amount of DNA. Here we use Whole Genome Amplification to develop microsatellite markers in reniform nematode for studying the variability of individual reniform nematodes. The objective of this research was to use polymorphic microsatellite markers to study the genetic variability of individual reniform nematodes from the southeastern cotton growing region.

## Materials and Methods

*Microsatellite development:* Reniform nematodes were obtained from infested field soil from Calhoun County, South Carolina and maintained in a greenhouse on cotton. DNA was extracted from 10 vermiform *R. reniformis* using 25µl Worm Lysis Buffer (KCl 50mM, Tris pH 8.3 10mM, Tween 20 0.45%, Triton X 0.45%, Proteinase K 60 µg/ml, $MgCl_2$ 2.5m, $dH_20$). Nematodes were placed directly into buffer in a 1.5 mL centrifuge tube and incubated at -80°C for 10 minutes. Nematodes were crushed with a pestle and the tube was incubated at 65°C for one hour. Tube was vortexed several times during incubation to help breakup nematode tissue. The tube was then incubated at 95°C for 15 minutes to inactivate the Proteinase K and subsequently cooled to 4°C. One µl of extracted DNA was used for Whole Genome Amplification (WGA) using GE Healthcare's GenomiPhi V2 kit and followed manufacturer's protocol.

Microsatellite DNA loci were then isolated following the protocol established by Glenn and Schable (2005). After WGA, two samples of 19 µl of DNA extract were digested with enzyme BstU I and two samples of the same amount of DNA were digested with enzyme RSA I (New England Biolabs). Restriction enzyme Xmn I (NEB) was also added at this point in time to avoid the dimerization of linkers. Following restriction, a 1% agarose gel was used to visualize 5 µl of product to confirm that DNA was digested resulting in a continuous smear less that 1,500 bp.

Restricted DNA was ligated to a double-stranded linker to provide the primer binding site for subsequent PCR. Forward SuperSNX24 (5' – GTTTAAGGCCTAGCTAGCAGAATC-3') and reverse SuperSNX24+4P (5'-

pGATTCTGCTAGCTAGGCCTTAAACAAAA-3') (IDT) were used. The phosphate on the reverse linker allowed for ligation of linkers to the digested DNA. Ten µl of double stranded SuperSNX linkers were added to 15 µl of restricted DNA. To ensure that linker ligation was successful, PCR amplification was performed on 2 µl of linker ligated DNA and visualized on a 1% agarose gel. A smear centered at approximately 500 bp was confirmed.

A mixture of 3' biotinylated oligos was used for enrichment of microsatellite containing DNA fragments. A mix of oligos at 1 µM each [$(AAAC)_6$, $(AAAG)_6$, $(AATC)_6$, $(AATG)_6$, $(ACAG)_6$, $(ACCT)_6$, $(ACTC)_6$, $(ACTG)_6$] was used as the microsatellite probe with 10 µl of linker ligated DNA. Fifty µl of streptavidin-coated magnetic Dynabeads (Dynal) were washed per DNA+probe mixture (200 µl total). All of the DNA+probe mix was added to the washed Dynabeads. Microsatellite-enriched DNA was removed from beads and amplified in polymerase chain reactions using SuperSNX-24 as a forward primer. A small portion of the product (4 µl) was visualized on a 1% agarose gel to verify that DNA recovery was successful.

DNA was then cloned using Invitrogen's TOPO TA Cloning Kit with PCR 4-TOPO vector. Transformation followed manufacturer's guidelines with 4 µl of PCR product used. Twenty-five µl of transformed bacteria were plated on LB plates containing 50 µg/ml ampicillin. Non-recombinant vector cells are killed upon plating thus eliminating the need for blue/white screening. Transformed colonies were picked and amplified using an MJ Research PTC-100 thermocycler with M13 forward (-20) (5'-

GTAAAACGACGGCCAG-3') and reverse (5'-CAGGAAACAGCTATGAC-3') primers. A 1% agarose gel was used to ensure the presence of inserts.

PCR products containing inserts within the 300 to 1,000 bp range were purified using equal volumes of Exonuclease I (NEB) and Antarctic Phosphatase (NEB). Purified DNA was sequenced using an ABI 3130xl DNA analyser located at Clemson University's Genomics Institute using M13 forward and reverse primers (forward 5'-GTAAAACGACGGCCAG-3', reverse 3'-GGCTATGACCATG-5'). Sequences from forward and reverse reads were assembled and edited using BIOEDIT incorporating the Clustal W alignment method. Mine SSR, a program available through the Clemson University Genomics Institute (http://www.genome.clemson.edu/resources/online_tools/ssr), was used to identify microsatellite repeats in contiguous sequences and design primers. After primers were designed to amplify *R. reniformis* microsatellite loci, sequences were analyzed using NCBI's Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to find regions of similarity with other nucleotide sequences.

*Microsatellite analysis of individuals:* Ten individuals from each of 15 locations and 10 individuals of *R. parvus* were included in this study and are considered the subpopulations in all subsequent analyses while all 160 individuals were considered as the total population. Isolation locations in the US included Alabama, Arkansas, Florida, Georgia, Louisiana, Mississippi, North Carolina, South Carolina, and Texas (Table 4.1).

*R. parvus* is a parthenogenetically reproducing species that was obtained from Arizona. Individuals from Colombia and Japan were also included in the study to determine genetic variability of isolates within the US compared to other countries. Two populations from Japan were included. One reproduced amphimictically (JA) and one that had been previously described as reproducing only by parthenogenesis (JP).

Whole cell content DNA was extracted from 160 individual reniform nematodes using Sigma Extract-N-Amp kit (XNAT2). To extract DNA from individuals, the manufacturer's protocol was modified by reducing all volumes to one eighth of the recommended amounts. One nematode was placed into a 0.2 ml centrifuge tube containing 12.5 µl Extraction Solution. The nematode was then crushed using the tip of a <10 µl pipette tip. Subsequently, 3.5 µl of Tissue Prep solution was added to the tube. The tube was then mixed and centrifuged briefly to collect contents. Tubes were then incubated at 65 °C for 10 minutes followed by 95 °C for 3 minutes. Next, 12.5 µl of Neutralization Solution B was added to each tube, mixed, and centrifuged briefly. Extracted DNA was then stored at 4 °C.

DNA from each individual was amplified at 10 polymorphic microsatellite loci. Optimized annealing temperature was determined for each microsatellite primer pair using an MJ Research PTC-100 thermocycler (Table 4.2). Cycling conditions were as follows: 94°C for 3 minutes followed by 35 cycles at 94°C for 30 seconds, optimum primer annealing temperature for 30 seconds, 72°C for one minute and a final extension at 72°C for 10 minutes. Each 20 µl PCR reaction contained 2 µl DNA extract and 18 µl

PCR mix (1.25X PCR Buffer, 0.25 mM dNTPs, 2.5 mM $MgCl_2$, 0.5 µM Forward Primer, 0.5 µM Reverse Primer, 0.125 U Taq). All forward primers were labeled with fluorescent dye FAM (Invitrogen). Amplicons were detected using an ABI 3730xl DNA Analyser with GeneScan 500 ROX used as the size standard (Applied Biosystems). Peak data was analyzed using Peak Scanner software version 1.0 (Applied Biosystems) and allele calls were visually inspected.

*Data Analysis:* The Excel Microsatellite Toolkit (Park, 2001) was used to determine polymorphism information content, number of alleles per locus, and observed and expected heterozygosities. GenePop V 4.0 (Rousset, 2008) was used to test for linkage between loci, deviation from expected Hardy-Weinberg proportions, distribution of genotypes, and Rho statistics. Arlequin V 3.5 (Excoffier & Lischer, 2010) was used to analyze F-statistics between subpopulations and within a subpopulation. Population structure was assessed using STRUCTURE V 2.3, which uses a Bayesian Approach to infer the most likely number of populations (K) present (Hubisz et al., 2009). STRUCTURE Tools for Population Genetic Analyses (TFPGA) V 1.3 (Miller, 1997) was used for cluster analysis based on unbiased distance between subpopulations.

## Results

Ten microsatellite loci developed proved to be polymorphic and were used to determine the genetic variability of individual reniform nematodes. From BLAST analysis of sequences, no other sequences in the nucleotide database were found to be significantly similar to microsatellite sequences using the megablast algorithm. Due to lack of significant similarity, no function of sequences could be inferred. Analysis of

allele sizes of 160 individual reniform nematodes at the 10 microsatellite loci developed resulted in total alleles per locus ranged from 10 to 24 (Table 4.2). Expected and observed heterozygosities ranged from 0.233 to 0.911 and 0.069 to 0.569, respectively. Polymorphism information content (PIC) ranged from 0.229 to 0.900. PIC (Botstein et al., 1980) is an index of how informative a genetic marker can be by taking into account the number of alleles and their frequencies and is measured by the probability that a parent is heterozygous multiplied by the probability that the offspring is informative. Loci were not restricted to amplification in *R. reniformis* but were also amplified in *R. parvus*.

From GenePop V 4.0 (Rousset, 2008), no significant linkage disequilibrium was found among any pairs of loci (*P*-value 0.01) using Fisher's method. At the 0.01 significance level, only one locus (RR2-6) was found to be in expected Hardy-Weinberg proportions for all 160 isolates when using a null hypothesis that assumes random union of gametes and the alternative hypothesis is a deficiency of heterozygotes. The distribution of diploid genotypes in populations was tested with the null hypothesis "genotypes are drawn from the same distribution in all populations" (GenePop V 4.0 option 3, sub-option 4) (Rousset, 2008) and results were based on contingency tables. Genotypes were found to not be drawn from the same distribution in all populations across all 10 loci and were considered significantly different (*P*-value 0.01) (Table 4.3). The rejection zone was defined as the sum of the probabilities of all tables having a log-likelihood ratio (*G*) higher than or equal to the observed *G* value. Analysis was performed for all pairs of samples across all loci. Based on individual genotypic

differentiation, the Japan parthenogenetic (JP) isolate was found to be significantly differentiated from all other isolation locations (*P*-value 0.01). The Louisiana isolate from soybean (LAS) was statistically differentiated from all other isolates, except from the other isolate from Louisiana found parasitizing cotton (LAC). A similar result was also observed for the isolate parasitizing tobacco from Colombia (CT) which was differentiated from other isolates except the other isolate from Colombia parasitizing banana (CB). This suggests that geographic origin has a greater effect than host on isolate differentiation. *Rotylenchulus parvus* was not significantly differentiated from all isolation locations.

Deficiencies of heterozygotes lead to a departure from expected Hardy-Weinberg genotype proportions. Therefore, analysis of Wright's F-statics was used to determine deviation in heterozygosity using Arlequin V 3.5 (Excoffier & Lischer, 2010). For our study, 10 individuals from a single isolation location were considered as a subpopulation while the total population was all 160 individuals. Results showed a mean $F_{IS}$ = 0.54087, $F_{ST}$ = 0.12935, $F_{IT}$= 0.60026 indicating that most of the deficiency in heterozygotes was a result of individuals relative to subpopulations and total population rather than a result of the subpopulation relative to the total population. A relative high value for $F_{IS}$ indicated high inbreeding within subpopulations. Subpopulations also showed a moderate genetic differentiation among populations based on $F_{ST}$ analysis. According to Nei (1978), $F_{ST}$ values between 0.05 and 0.15 indicate moderate genetic differentiation, values between 0.15 and 0.25 indicate a great differentiation, and values above 0.25 indicate a very great differentiation. No $F_{ST}$ values were found to be negative. Pairwise $F_{ST}$ values (Table

4.4) indicated that all isolates were a minimum of greatly differentiated genetically from JP and exhibit a high proportion of genetic diversity from allele frequency differences among the populations relative to JP. *R. parvus* (RP) was only moderately genetically differentiated from most isolates except North Carolina (NC) and Texas (TX). Based on allele frequency differences, NC and TX did not exhibit genetic differentiation from RP due to subdivision. Georgia (GA) and LAC showed the least amount of differentiation from other isolation locations excluding JP.

Rho statistics were calculated using GenePop V 4.0 (Rousset, 2008) which are based on allele sizes and not number of repeats. These measures are analogous to Wright's F-statistics and showed a similar result to F-statistical analysis. The overall analysis resulted in a mean $\rho_{IS} = 0.7439$, $\rho_{ST} = 0.2095$, $\rho_{IT} = 0.7975$. A relatively high value for $\rho_{IT}$ across loci also indicated an excess in homozygotes that is usually related to a high inbreeding effect between individuals within isolation locations. The fixation index of differentiation between populations division ($\rho_{ST}$) shows that most of the variation is due to genetic differences within isolation locations (80%) rather than between isolation locations (20%).

TFPGA (Miller, 1997) was used to determine genetic distances between isolation locations and to illustrate a dendrogram of isolate clustering. All UPGMA clustering algorithms placed the Japan parthenogenetic population as the basal population 100% of the time at all 10 loci. Other nodes did not have a high bootstrap support value which is a measure of support strength for nodes in phylogenetic tree analysis which measures the

resolve of a specific clade. Nei's 1972 minimum distance measure was used for all subsequent UPGMA cluster analyses because zero trees produced from bootstrap sampling contained ties and proportion of similar replicates was higher per node when compared with other distance measures. One node contained what was considered a significant bootstrap value (above 80%) which separated JP from all other isolation locations (Figure 4.1). The genetic distance was calculated to be 0.2267. UPGMA clustering failed to parse out RP from other isolation locations indicating that the species is still closely related to *R. reniformis*.

A no admixture model with correlated allele frequencies was used in STUCTURE V 2.3 (Hubisz et al., 2009) with a 100,000 burn-in period followed by 500,000 MCMC iterations. The model was chosen as the best fit due to a small α value (<0.09) being returned when analyzing data assuming an admixture model. The manual suggests that if a small α value (near zero) is calculated using an admixture model, then most individuals have little admixture. Our results returned α values 0.04 to 0.06 when K was varied from 2 to 20. A likelihood ratio test was used to determine whether a model with allele frequencies correlated or independent was the best fit. From the likelihood ratio test, a correlated allele frequency was found to be the best fit model. The value of K was varied from 2 to 20. The value with the highest log likelihood value was K=14 (Ln P(D)=-3433.72). Analysis did not reveal a distinct cluster of isolates based on isolation (Figure 4.2). Only the individuals isolated from Japan that reproduced parthenogenetically were parsed out as a distinct isolation location. Again, *R. parvus* was not found to be a distinct group of individuals, but alleles present in the haplotypes were also observed in

haplotypes from *R. reniformis* isolates. JP had the highest distinct Bayesian cluster compared with other isolation locations of 94.1%. The only other location that had above 5% clustering with JP's distinct cluster were isolates from GA with 12.5% homogeneity to the JP isolate. The isolates from GA exhibited less than 17% homogeneity to any of the 14 clusters suggested that the isolates from GA have maintained many alleles that can still be found in other isolation locations. The next highest cluster was assigned to CT of 85.5% with the third highest being LAS with 72.4% homogeneity. TXF was the only other isolation location to exhibit above 50% (52.7%) homogeneity of isolates based on cluster analysis.

Discussion

Reniform nematode populations appear to be able to maintain a high level of genetic diversity at 10 microsatellite loci. This high level of genetic diversity did not allow a correlation of haplotype to isolation location. The only distinct isolation location was the Japan parthenogenetic (JP) population, supporting the fact that this isolate constitutes a different species. The parthenogenetically reproducing isolate from Japan was originally described as *Tetylenchus nicotiana* (Yokoo & Tanaka, 1954), was later moved to the genus *Rotylenchulus* by Baker (1962), and then re-described as *R. nicotiana* by Nakasono and Ichinohe (1967). In 1968, Dasgupta et al. synonymized *T. nicotiana* (*R. nicotiana*) with *R. reniformis* along with four other species (*R. elisensis*, *R. queirozi*, *R. leiperi*, and *R. stakmani*). Nakasono (2004) suggested reproductive isolation between male-numerous isolates from Japan and male-rare or male-absent types. For our study

the male-absent type was represented by isolate JP. The presence of males was suggested to be genetically determined not environmentally. Agudelo et al. (2005) found 11.78% divergence in ITS1 sequences from this isolate when compared to 20 amphimicticallly reproducing populations of *R. reniformis*. In this study, the only population that was distinguishable based on ITS1 sequence analysis was a parthenogenetic population from Japan. Sequences were conserved for all amphimictically reproducing isolates but isolates from Limestone, AL, Huxford, AL, Arkansas, Georgia, and Texas were distinguishable based on morphometrics and/or host preferences.

Based on TFPGA and STRUCTURE population grouping analysis, JP was more distant genetically from *R. reniformis* isolates than *R. parvus* at 10 microsatellite loci studied. Greater genetic distance can cause a lower percentage of loci to amplify between species (Jarne & Logoda, 1996), but all 10 loci were amplified in both *R. reniformis* and *R. parvus* suggesting that both species are very closely related. STRUCTURE analysis revealed that many isolates share alleles but there is not a distinct haplotype that correlates with isolation location or host. More alleles appear to be shared between amphimictically reproducing *R. reniform*is isolates and *R. parvus* than are shared with JP. The alleles present in *R. parvus* were not found in all isolation locations but may represent the presence of ancestral alleles.

A previous microsatellite study of reniform nematode populations (Arias et al., 2009) used DNA pooled from 1,000 to 2,000 eggs rather than DNA extracted from individuals. Due to high intraspecific variation among individuals within sampling

locations, pooled nematode DNA is not a suitable method for characterizing variation. Arias et al. (2009) found differences between isolates from four southern cotton growing states: TX, LA, MS and GA. Since there was high variation between individuals from one sampling location, pooled DNA may not show the true amount of variation present from one sampling location. Based on F-statistics, deficiency of heterozygotes was a result of the individual and not the subpopulation.

Previous work on variability in response to temperature during embryogenesis in reniform nematode has been reported among isolates from Alabama, Mississippi, and South Carolina (Leach et al., 2009). These isolates were also included in this study to determine if genetic variation between isolates could be correlated with phenotypic variation previously observed. From loci studied, no genetic variation could be associated with differences in biology. There was no significant genotypic differentiation based on genotype distribution among isolates (Table 4.3). Analysis of $F_{ST}$ resulted in Mississippi and Alabama having moderate differentiation (Table 4.4), but this value relatively low (0.088).

Isolates from Texas showed subdivision and genetic differentiation between individuals cultured in a greenhouse versus individuals retrieved from a field (TXF). When comparing $F_{ST}$ values, which is a measure of the deficiency in heterozygotes of the subpopulation relative to the total population, the two isolates were highly differentiated ($F_{ST}$=0.1587). In contrast, isolates parasitizing different hosts from Colombia and Louisiana did not show population subdivision or genetic differentiation. Both isolates

from Colombia and Louisiana were obtained from an infested field and were not cultured in a greenhouse. These observations support that isolates maintained in a greenhouse will exhibit differences in genetic diversity when compared to field population.

An excess of homozygotes was observed at all isolation locations resulting in isolates not conforming to expected Hardy-Weinberg proportions. The most common cause of homozygote excess is nonrandom mating or the Wahlund effect. The Wahlund effect is often due to population subdivision when two distinct populations are analyzed as one (Allendorf & Lundquist, 2007). The presence of null alleles can also cause a perceived deficiency of heterozygotes.

The Wahlund effect is the result of subpopulations having different allele frequencies being studied as one population, but when studied individually, are in Hardy-Weinberg proportions. Our results do not indicate a Wahlund effect occurring at each isolation location. Each isolation location was studied separately and none were found to be in expected Hardy-Weinberg proportions. Also, the excess of homozygotes observed was neither locus specific nor population specific again supporting that our results were not due to a Wahlund effect but that another factor was causing the excess in homozygotes.

Null alleles are common in genetic studies in DNA markers such as microsatellites (Brookfield, 1996; Paetkau & Strobeck, 1995; Pemberton et al., 1995). From our results, there is relatively little variance in $F_{IS}$ across loci. Also, loci with the highest $F_{IS}$ variance were not those with the highest calculated $F_{IS}$ (Figure 4.3), which

would be expected with the presence of null alleles. These results make the null allele explanation unlikely.

Based on $F_{IS}$, the excess of homozygotes is most likely due to a high level of inbreeding within isolation locations. Here $F_{IS}$ is the measure of heterozygote deficiency of individuals relative to the subpopulation or isolation location where they were obtained. Due to the relatively limited mobility of *Rotylenchulus* sp. an excess in homozygotes due to inbreeding is a likely scenario. Brothers and sisters from a single egg mass are likely to procreate due to relatively limited mobility.

An excess of heterozygotes is often expected when studying parthenogenetically reproducing populations such as JP and *R. parvus* and is indicated by a strongly negative $F_{IS}$ value (Balloux et al., 2003). Our results did not show an excess in heterozygotes at 10 microsatellite loci for any isolation location. A similar result was observed by Rougeron et al. (2009) when studying 124 strains of *Leishmania braziliensis* from 4 hosts at 12 microsatellite loci. All loci exhibited a positive value for $F_{IS}$ even though *L. braziliensis* has historically been considered a clonally reproducing species. The high level of homozygotes may also be an effect of isolates being introduced to the areas where they were collected. Introduced species often exhibit a substantial loss of genetic diversity when they have originated from a small founder population causing a severe founder effect and genetic drift (Allendorf & Lundquist, 2003; Sakai et al., 2001).

UPGMA cluster analysis and STRUCTURE analysis did not reveal distinct clustering for various reniform nematode isolates due to geographic origin or host

resulting in an unresolved phylogenetic analysis (Figure 4.1). The high variation present in the *Rotylenchulus* isolates along with sharing of alleles between isolation locations may explain the uninformative phylogeny. Phylogenetic analysis may not be possible if conflict arises when multilocus data are combined or when multiple mutations have occurred at the same site (Machado & Hey, 2003). The individual gene genealogy may have distinct and possibly incompatible evolutionary histories. More microsatellite markers may be needed or a different type of marker may allow for parsing out of isolates based on isolation location or host.

The high level of variation does not correlate to host or locality, resulting in a wide variety of genetic variability between isolates. A similar result was found for *Fusarium poae* isolates from Argentina and England (Dinolfo et al., 2010) when using inter-simple sequence repeats. Isolates did not show a clear relationship which could be associated with host or geographic origin. Isolates with the same haplotype were collected from different geographic regions. Maintenance of a high level of genetic variation can indicate the potential for population adaptability. Dennehy et al. (2010) showed that a genetically shifting source population can fuel evolution and potential disease emergence. Genetic variation can lead to adaptive potential and increased distribution of a species. We have shown that *R. reniformis* is a highly variable species at 10 microsatellite loci. Characterizing variants could aid management strategies by allowing the ability to monitor changing populations of reniform nematodes that can be correlated to useful traits such as resistance-breaking, increased fitness, or higher virulence.

**Table 4.1**. Description of *Rotylenchulus* isolates.

| Species | Location | Host | Abbreviation |
|---|---|---|---|
| *R. reniformis* | Alabama | Soybean | AL |
| *R. reniformis* | Arkansas | Soybean | AR |
| *R. reniformis* | Colombia | Banana | CB |
| *R. reniformis* | Colombia | Tobacco | CT |
| *R. reniformis* | Florida | *Dracaena* spp. | FL |
| *R. reniformis* | Georgia | Cotton | GA |
| *R. reniformis* | Japan Amphimictic | Sweet Potato | JA |
| *R. reniformis* | Japan Parthenogenetic | Sweet Potato | JP |
| *R. reniformis* | Louisiana | Cotton | LAC |
| *R. reniformis* | Louisiana | Soybean | LAS |
| *R. reniformis* | Mississippi | Soybean | MS |
| *R. reniformis* | North Carolina | Soybean | NC |
| *R. reniformis* | South Carolina | Cotton | SC |
| *R. reniformis* | Texas Field | Cotton | TXF |
| *R. reniformis* | Texas Greenhouse | Cotton | TX |
| *R. parvus* | Arizona | Bentgrass | RP |

**Table 4.2** Primer sequences and characteristics of 10 polymorphic microsatellite loci amplified in 160 *Rotylenchulus* individuals from various hosts and isolation locations.

| Locus | Repeat motif | Primer sequence (5' → 3') | $T_a$ (°C) | $k$ | $H_e$ | $H_o$ | PIC | $F_{IS}$ | GenBank Accession no. |
|---|---|---|---|---|---|---|---|---|---|
| RR1-5 | $(ga)_7$, $(ga)_7$, $(ga)_7$, $(ga)_7$ | F:TGCGAGAGAG AGAGAGAGTGA R:TGGTGTTAAT GGTGAAAGCG | 62 | 24 | 0.84 | 0.38 | 0.82 | 0.529 | HQ158019 |
| RR2-5 | $(cact)_3$ | F:GGGCACTGAA AACACCAAGA R:TGAGTGGACA CAATCCGAAA | 60 | 11 | 0.51 | 0.07 | 0.45 | 0.816 | HQ158020 |
| RR2-6 | $(catt)_4$ | F:GAATGAATTG GCAAAATGGC R:CTCCATGGCTT TCTCCTCAG | 62 | 12 | 0.65 | 0.57 | 0.59 | 0.079 | HQ158021 |
| RR3-3 | $(catt)_3$, $(attc)_3$ | F:AATGGGCGTC TAGTTCCCTT R:ATCGAAAATG ACATGCACCA | 60 | 17 | 0.88 | 0.37 | 0.87 | 0.652 | HQ158013 |
| RR3-8 | $(ga)_{10}$, $(ag)_5$, $(ag)_5$ | F:TTGCTTTACTT CCGCTTCGT R:GCTCCATTTTT CCCACTCAA | 61 | 22 | 0.80 | 0.47 | 0.78 | 0.300 | HQ158015 |
| RR4-1 | $(ttga)_4$ | F:AGAAAATGCT TTTCCCGAGG R:AGAATTAGCG TTTGGGGATG | 63 | 17 | 0.67 | 0.23 | 0.65 | 0.501 | HQ158016 |
| RR4-4 | $(ttga)_4$ | F:GAAAATGCTTT TCCCGAGGT R:CGGTAGAATT AGCGTTTGGG | 63 | 10 | 0.54 | 0.22 | 0.51 | 0.442 | HQ158017 |
| RR4-5 | $(tggg)_3$ | F:TGTAATGACCC GAGTGTGGA R:GGGTCTCCCG GTAGTACGAT | 57 | 13 | 0.72 | 0.29 | 0.70 | 0.657 | HQ158018 |
| RR5 | $(ttg)_4$ | F:CTCCGCCATTA CCATTCTGT R:CCCCAATTCC AACAACAATC | 54 | 23 | 0.91 | 0.23 | 0.90 | 0.829 | GU471239 |
| RR2 | $(tggg)_3$ | F:AAAGATGCCA AAAGAATGCG R:GATATGCGTT GGCTGTGTTG | 58 | 13 | 0.23 | 0.09 | 0.23 | 0.561 | GU471236 |

$T_a$ is the PCR annealing temperature; $k$ is the number of alleles observed at each locus; $H_e$ and $H_o$ are expected and observed heterozygosities, respectively, PIC is polymorphic

information content at each locus, inbreeding coefficient ($F_{IS}$) and the GenBank Accession number.

**Table 4.3.** Genotypic differentiation between subpopulations (*P*-value = 0.01) based on genotype distributions.

| | AL | AR | CB | CT | FL | GA | JA | JP | LAC | LAS | MS | NC | SC | TXF | TX | RP |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AL | - | | | * | | | * | * | | * | | * | | * | * | * |
| AR | | - | * | * | * | * | * | * | * | * | * | * | | * | * | * |
| CB | | | - | | | * | | * | * | * | * | * | | * | * | * |
| CT | | | | - | * | * | * | * | * | * | * | * | * | * | * | * |
| FL | | | | | - | | * | * | | * | * | | | * | | |
| GA | | | | | | - | | * | | * | | | | * | | |
| JA | | | | | | | - | * | | * | * | * | * | | * | * |
| JP | | | | | | | | - | * | * | * | * | * | * | * | * |
| LAC | | | | | | | | | - | | | | * | * | | |
| LAS | | | | | | | | | | - | * | * | * | * | * | * |
| MS | | | | | | | | | | | - | | | * | * | * |
| NC | | | | | | | | | | | | - | * | * | * | |
| SC | | | | | | | | | | | | | - | * | | * |
| TXF | | | | | | | | | | | | | | - | * | * |
| TX | | | | | | | | | | | | | | | - | |
| RP | | | | | | | | | | | | | | | | - |

* Indicates a significant result and rejects the null hypothesis "genotypes are drawn from the same distribution in all populations".

Definition of abbreviations: AL-Alabama, AR-Arkansas, CB-Colombia (host banana), CT-Colombia (host tobacco), FL-Florida, GA-Georgia, JA-Japan (reproduces amphimictically), JP-Japan (reproduces parthenogenetically), LAC-Louisiana (host cotton), LAS-Louisiana (host soybean), MS-Mississippi, NC-North Carolina, SC-South Carolina, TXF-Texas (isolated from cotton field), TX-Texas (maintained in a greenhouse on cotton), RP-*Rotylenchulus parvus*

**Table 4.4**. Pairwise $F_{ST}$ between isolation localities of *Rotylenchulus reniformis* and one *Rotylenchulus parvus* isolate.

| | AR | CB | CT | FL | GA | JA | JP | LAC | LAS | MS | NC | SC | TXF | TX | RP |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AL | 0.017 | 0.072* | 0.175** | 0.071* | 0.024 | 0.118* | 0.226** | 0.050* | 0.160** | 0.088* | 0.116* | 0.040 | 0.187** | 0.124* | 0.097* |
| AR | - | 0.134* | 0.230** | 0.081* | 0.070* | 0.168** | 0.265*** | 0.061* | 0.143* | 0.105* | 0.125* | 0.054* | 0.200** | 0.090* | 0.096* |
| CB | | - | 0.044 | 0.090* | 0.075* | 0.060* | 0.168** | 0.102* | 0.196** | 0.183** | 0.152** | 0.096* | 0.152** | 0.169** | 0.158** |
| CT | | | - | 0.218** | 0.133* | 0.070* | 0.202** | 0.171** | 0.262*** | 0.263*** | 0.247** | 0.210** | 0.179** | 0.256*** | 0.252*** |
| FL | | | | - | 0.088* | 0.173** | 0.241** | 0.059* | 0.193** | 0.138* | 0.097* | 0.081* | 0.190** | 0.085* | 0.098* |
| GA | | | | | - | 0.042 | 0.165** | 0.048 | 0.125* | 0.053* | 0.062* | 0.057* | 0.097* | 0.094* | 0.050* |
| JA | | | | | | - | 0.123* | 0.089* | 0.170** | 0.201** | 0.143* | 0.168** | 0.060* | 0.190** | 0.179** |
| JP | | | | | | | - | 0.190** | 0.270*** | 0.284*** | 0.196** | 0.275*** | 0.192** | 0.254*** | 0.236** |
| LAC | | | | | | | | - | 0.041 | 0.098* | 0.097* | 0.073* | 0.136* | 0.076* | 0.068* |
| LAS | | | | | | | | | - | 0.164** | 0.161** | 0.129* | 0.149* | 0.094* | 0.127* |
| MS | | | | | | | | | | - | 0.090* | 0.044 | 0.208** | 0.111* | 0.094* |
| NC | | | | | | | | | | | - | 0.098* | 0.118* | 0.074* | 0.040 |
| SC | | | | | | | | | | | | - | 0.176** | 0.061* | 0.073* |
| TXF | | | | | | | | | | | | | - | 0.159** | 0.144* |
| TX | | | | | | | | | | | | | | - | 0.045 |

*Indicates moderate genetic differentiation, 0.05-0.15

** Indicates high differentiation, 0.15-0.25

***Indicates very high differentiation, >0.25

Definition of abbreviations: AL-Alabama, AR-Arkansas, CB-Colombia (host banana), CT-Colombia (host tobacco), FL-Florida, GA-Georgia, JA-Japan (reproduces amphimictically), JP-Japan (reproduces parthenogenetically), LAC-Louisiana (host cotton), LAS-Louisiana (host soybean), MS-Mississippi, NC-North Carolina, SC-South Carolina, TXF-Texas (isolated from cotton field), TX-Texas (maintained in a greenhouse on cotton), RP-*Rotylenchulus parvus*

**Figure 4.1**. UPGMA dendrogram of *Rotylenchulus* isolates from 10 microsatellite loci constructed using Nei's (1972) minimum distance measure of genetic diversity. Numerical values of nodes with bootstrap values of less than 80% are not shown as they were not considered significant.

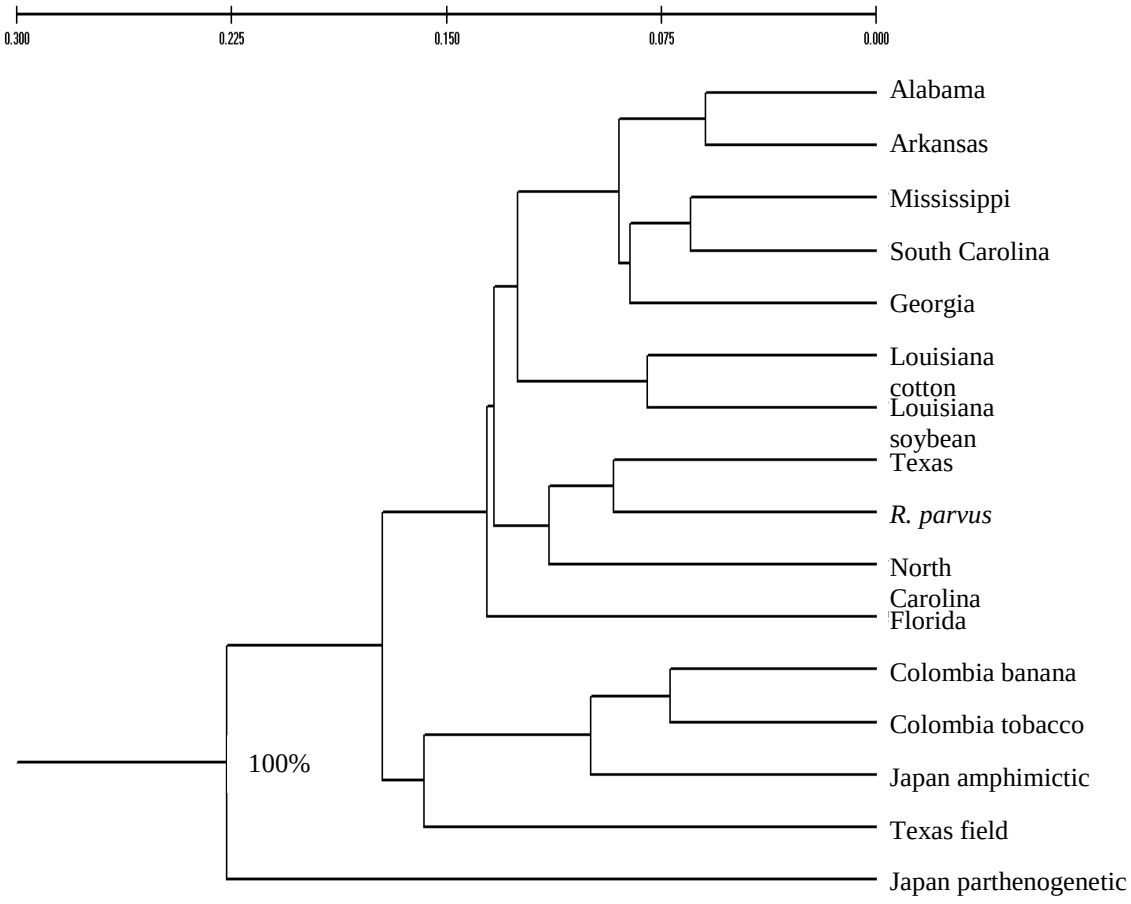


**Figure 4.2**. Graphical representation of haplotype similarity assignments at 10 microsatellite loci for 160 *Rotylenchulus* individuals at the best fit grouping number K=14. Each color represents a cluster based on alleles present in an individual's haplotype. Each line represents the haplotype of an individual at 10 microsatellite loci.

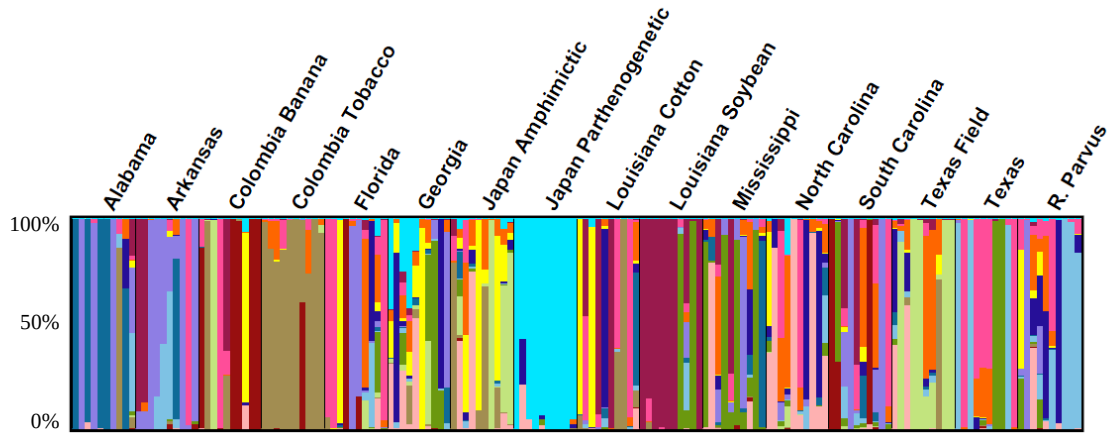
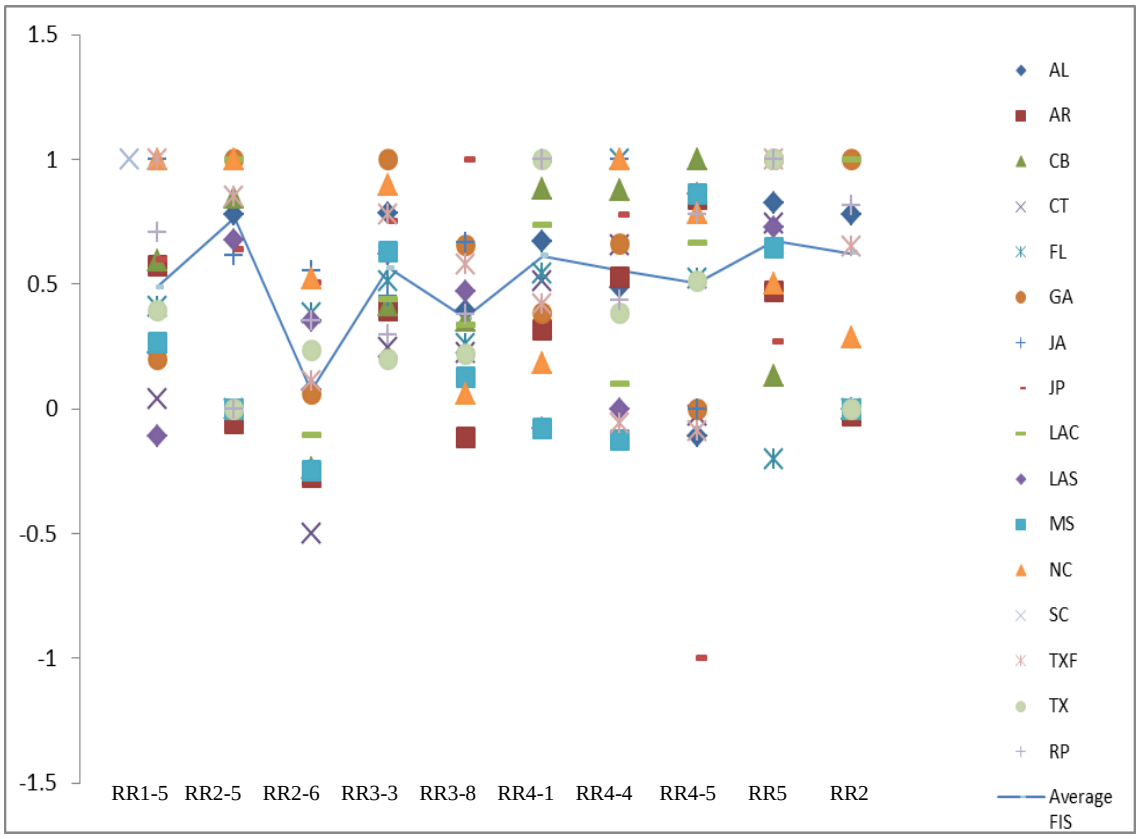


**Figure 4.3**. $F_{IS}$ for 10 microsatellite loci in 16 *Rotylenchulus* subpopulations and average $F_{IS}$ for each microsatellite loci exhibiting a large heterozygote deficiency at each locus.



# CHAPTER V

## CONCLUSIONS AND FUTURE RESEARCH

Reniform nematode variability has been studied by using AFLP markers to analyze host selection pressure on a population, by measuring differences in embryogenesis between geographic isolates, and by determining the genetic variability of geographic isolates.

Crop rotations exert selection pressure on a population and alter the predominant genotype. The most distinct effects were observed when resistant or susceptible soybean cultivars were used in a rotation scheme. Continuous cotton cropping appears to maintain diversity of a population's genotype but allows for genetic drift from the original field genotype. Continuous soybean maintains the original field genotype for a longer period of time. These results suggest that the use of susceptible soybean cultivar as a host may be more useful for maintaining field genotype when culturing reniform nematodes in a greenhouse. There was an observed effect of host selection due to crop rotations.

Differences in embryonic development in response to temperature are another measure of variability of reniform nematode. Three populations isolated from cotton fields in the Southeastern United States were observed for this response at temperatures ranging from 20 to 35°C. A correlation between average temperatures of geographic origin of isolates and optimal temperature for embryogenesis was observed for two out of three isolation locations. One isolate was able to maintain a steadier hatching rate as

temperatures increased, showing a difference in plasticity of the species in regard to isolation location. Differences were observed at the temperature extremes (20 and 35°C) of the study while time to hatch was similar in the middle range (25 to 30C°) among the three isolates. These results show that there are developmental differences in response to temperature among geographic isolates that can be the result of high (35°C) and low (20°C) temperatures. Reniform nematode populations present in the cotton-growing region of the United States have different abilities to respond to variations in temperature which may be the result of genetic variability between isolation locations.

To determine the genetic variability of *Rotylenchulus reniformis*, DNA from 10 individuals from 15 isolation locations and DNA from 10 *Rotylenchulus parvus* individuals from Arizona was amplified at 10 microsatellite loci. Reniform nematode populations appear to be able to maintain a high level of genetic diversity at the 10 microsatellite loci used in the study. This high level of genetic diversity did not allow a correlation of haplotype to geographic origin or host. The only distinct isolation location was a parthenogenetically reproducing isolate from Japan that had previously been described as a different species. Further analysis also showed the parthenogenetically reproducing isolate from Japan was more distant genetically from *R. reniformis* isolates than *R. parvus* at the 10 microsatellite loci studied.

Due to the high level of variability observed in reniform nematode populations, a standardized method for characterizing variants would be useful. Molecular markers like AFLPs or microsatellites may be a useful platform in which to start looking for molecular

markers that track genetic changes in a population's response to host or environment. Characterizing variants could aid management strategies by allowing the ability to monitor changing populations of reniform nematodes that can be correlated to useful traits such as resistance-breaking, increased fitness, or higher virulence. Reniform nematode populations may have the ability to increase distribution range through variants able to reproduce in a wider temperature range or on hosts previously considered as resistant.

A more thorough understanding of reniform nematode population responses to host plants will assist utilization of crop-mediated management options. Stable resistance genes need to be identified to avoid selection for host specific pathotypes able to overcome and reproduce well on host previously considered resistant. Assessment of long-term host-induced selection in the field can help determine whether markers are useful in resistance management. Genetic variability of isolates in response to crop rotations needs to be further studied in order to understand an isolate's response to selection pressure. Research aimed at identifying molecular markers able to discriminate a population's response to crop rotation or use of resistance can help to extend the durability of resistance and avoid emergence of isolates able to reproduce on what has traditionally been considered a non-host or poor host. Due to the high level of intraspecific variability observed at 10 microsatellite loci, future research for *R. reniformis* would need to include various geographic isolates to determine whether response to host selection pressure is similar across the species or varies by genotype.

Genetic variation and temperature response variation can indicate the potential for population adaptability. A genetically shifting source population can fuel evolution and potential disease emergence and can lead to increased geographic distribution of a species.

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